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Polypyrimidine tract binding protein (PTB) associates with intronic and exonic domains to squelch nuclear export of unspliced RNA

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ABSTRACT

Retention of unspliced pre-messenger RNA (pre-mRNA) in the nucleus is essential for cell survival. Available nuclear factors must recognize and discern between diverse export signals present in pre-mRNA to establish an export inhibitory complex. We found that polypyrimidine domains present in both intron and exon were important for export inhibition of a minigene transcript based on hepatitis B virus pregenomic RNA. Overexpression of PTB drastically reduced export and presence of RRM4 domain seemed critical. This inhibitory network overrode stimulation from an exonic export-facilitating element. We posit that binding of PTB to multiple loci on pre-mRNA regulates nuclear retention.

1. Introduction

Transport of macromolecules between nuclear and cytoplasmic compartments poses a significant challenge in eukaryotic cells. Even though our initial understanding of nuclear transport started with insight from protein trafficking, we now appreciate that movement of ribonucleoprotein (RNP) complexes is an important part of regulation of gene expression [1]. While different types of RNAs employ diverse mechanisms and dynamics of transport, mRNAs contain sequences that bind nuclear export factors to facilitate their passage through the nuclear pore complex [2]. However the recruitment of export factors to the unspliced pre-mRNA is actively regulated to ensure that they are retained within nucleus [3]. On the other hand, several viral pre-mRNAs harbor specific elements that aid in escaping nuclear surveillance. The constitutive transport element (CTE) of Mason-Pfizer monkey virus (MPMV) [4] and Rev Response Element (RRE) of human immunodeficiency virus [5] are good examples of such viral elements. While the MPMV-CTE employs the Tap/NXF1 pathway, the major channel of nuclear export of mRNA [6], the HIV-RRE is recognized by virally encoded Rev protein that eventually facilitates a Crm1 dependent nuclear exit [7].

In human hepatitis B virus (HBV), the unspliced pregenomic RNA (pgRNA) needs to leave the nucleus for translation and reverse transcription in the cytoplasm. Even though the pgRNA contains a number of canonical 5′ and 3′ splice sites, only the unspliced pool can provide the correct reading frame for translation of viral proteins. These transcripts accumulate late in infection after synthesis of sxRNA but prior to lx and pre-S1/pre-S2/S RNAs [8]. A *cis*-acting element termed Post-transcriptional Regulatory Element (PRE), has been linked to nuclear export of HBV subgenomic RNAs [9]. When put in heterologous reporter constructs, PRE behaved differently from MPMV-CTE as it could export cDNA copies of cellular genes [10] but did not support the export of intron-containing retroviral mRNAs [11]. Leptomycin B, an inhibitor of Crm1 mediated export, also could not prevent export of PRE containing transcripts [12]. It also differed from HIV-RRE in its independence from involvement of viral proteins. However PRE failed to stimulate viral pgRNA export but rather contributed to its stability [13]. PRE could bind to two cellular proteins, viz. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [14] and polypyrimidine tract binding protein (PTB) [15].

In mammalian systems, PTB was initially recognized as a global splicing repressor by binding to intronic splicing silencers, ISS [16]. However in-depth global profiling of PTB binding sites vis-à-vis repression or activation of splicing indicates a position-dependent splicing regulatory function in PTB [17]. Apart from splicing, other RNA maturation pathways are also regulated by PTB [18,19]. Generally, the specific pyrimidines contacted by PTB are present within an extended pyrimidine stretch and multiple such binding sites are present across the pre-mRNA [20]. The interaction between PTB molecules bound to upstream and downstream sequences may

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result in a zone of silencing by forming loop structure or by covering the RNA by multimerization [21,22]. To our knowledge interaction of PTB with RNA export apparatus has not been investigated carefully.

Here we report that interactions between PTB and different RNA elements affected nuclear export of a minigene pre-mRNA. We found that presence of polypyrimidine domains both in intron and exon was necessary to retain the pre-mRNA in nucleus. Presence of RRM4 domain in PTB was critical in blocking export even when these polypyrimidine sites were removed. The PTB driven inhibitory axis quenched an exonic export promoting function. We conclude that a negative regulation arising out of association of PTB molecules bound to polypyrimidine stretches in intron and exon counteract export stimulatory function present in PRE.

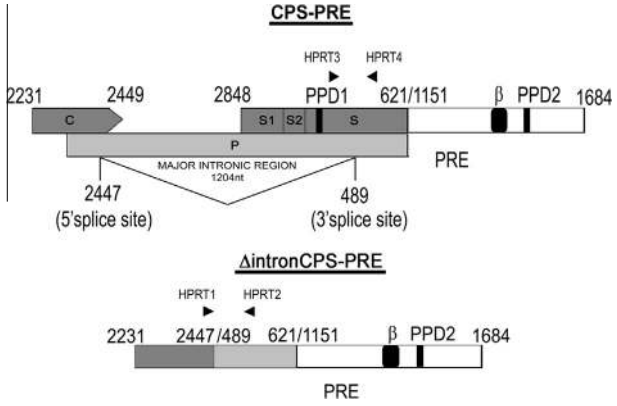

Fig. 1. Description of the minigene. The minigene construct (CPS-PRE) with major 5′ and 3′ splice sites, polypyrimidine domains (PPD), the export promoting domain β and PRE, is depicted. Arrowheads show location of primers used for quantitative RT-PCR amplification [23]. Numbers represent location in HBV genome (NCBI accession no. AY945307) shown here with encoded proteins (C,P.S1,S2,S). ΔintronCPS-PRE depicts the intronless copy of the same transcript.

2. Results and discussion

2.1. Description of the minigene

HBV utilizes cellular machinery for synthesis and maturation of viral RNAs. This presented us with an opportunity to study the interaction between nuclear factors and pre-mRNA. We constructed a minigene (CPS-PRE) based on HBV pgRNA with intact HBV intron but shortened exons (Fig. 1). We reason that such minigenes are useful for identification and understanding of role of individual elements and their interacting partners as has been done in numerous reports on RNA maturation pathways over the last two decades. In CPS-PRE minigene, upstream exon contained 215nt of endogenous sequence preceding the 5′ splice site and the 3′ exon had 131nt following the acceptor splice site to allow for short-range *cis* and *trans* interactions required for recognition of splice junctions. PRE was inserted in 3′ exon. The regulation of splicing in these constructs is already reported [23]. A similar construct with a cDNA copy of the same RNA was used to understand the effect of a complete intron on nuclear export.

2.2. Multiple pre-mRNA signatures collaborate to inhibit export

The minigene constructs were transfected in HuH7 hepatocarcinoma cell lines and nuclear and cytosolic RNAs were isolated. We calculated the extent of nuclear export of unspliced minigene transcript by quantitative RT-PCR on isolated nuclear and cytosolic fractions after normalization against U6 snRNA. We used U6 snRNA as control since it is exclusively nuclear and its aberrant cytosolic presence was used for rectification of sample preparation errors. We found that cytoplasmic:nuclear ratio of pre-mRNA from CPS-PRE was 0.58. Cytoplasmic accumulation of any particular minigene pre-mRNA was expressed relative to that of CPS-PRE to accurately depict fold difference in export. We found that either single or simultaneous alteration of the splice sites results in a moderate increase of export compared to the basal construct (Fig. 2A) indicating a role for 'intron definition' in pre-mRNA

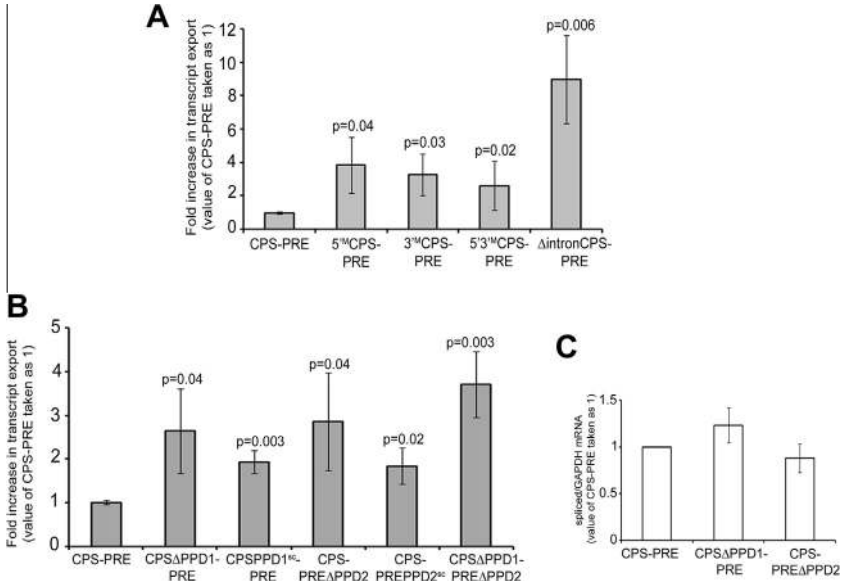

Fig. 2. Several factors contribute to pre-mRNA nuclear retention. (A) Intron definition and intronic sequences repress export. The 5′ splice donor site was changed from *GU* to *AU* and the 3′ splice acceptor site *AG* was mutated to *AC*. Quantitative RT-PCR experiments show that mutation of the 5′(5′ᴹ) and 3′(3′ᴹ) splice sites or absence of intron (ΔintronCPS-PRE) abrogated nuclear retention. (B) Deletions (ΔPPD1 and ΔPPD2) or changing specific pyrimidines to purines (PPD1ˢᶜ and PPD2ˢᶜ) increased nuclear export. Deletion of both (CPSΔPPD1–PREΔPPD2) showed better export indicating synergism between these domains. (C) Absence of PPD1 and PPD2 sites did not affect cytosolic spliced mRNA level. These sites are more relevant for export than splicing (also see Fig. 4C). All values are plotted relative to CPS-PRE from three independent quantitative RT-PCR experiments ± standard deviation.

retention. Interestingly the intronless copy of the same transcript was exported very efficiently. The sharp increase observed in the intronless copy over the constructs with mutant splice sites indicated that intronic sequences also took part in pre-mRNA retention. However complete absence of intron did not make the minigene pre-mRNA exclusively cytosolic. Therefore both intronic elements and intron definition are necessary but not sufficient for pre-mRNA nuclear retention.

We next localized the polypyrimidine domains (PPD) in both intron and PRE regions to understand this control [24,25]. Using the web-based RegRNA tool [26], the entire minigene sequence (CPS-PRE) was scanned for presence of pyrimidine rich PTB binding elements. We located two candidate polypyrimidine domains and named them PPD1 (246–251nt:UUCUCU in the intronic region) and PPD2 (1481–1488nt:CUCUCUCGU within PRE in 3′ exon). The PPD2 domain sits upstream of the other already documented polypyrimidine domains in PRE [15]. We explored whether PTB could bind to these domains and how far these interactions were important for regulation of export.

The deletion of PPD1 or substitution with a purine-rich sequence (PPD1^{sc}, UUCUCU::AAGAGA) showed increased export of pre-mRNA transcripts. Similar perturbation of the PPD2 site (PPD2^{sc}, CUCUCUCGU::AGAGGAAAG) also had the same effect (Fig. 2B). Simultaneous deletion of both PPD1 and PPD2 sites showed better export than single deletions. Our data suggest that multiple PTB nucleation sites on pre-mRNA are required for its retention. We did not observe any diminution in cytosolic abundance of spliced mRNA (Fig. 2C) or in the extent of splicing in the deletion constructs (Fig. 4C). These observations negate the possibility that enhanced export of pre-mRNA was an indirect effect of nuclear overload of unspliced pre-mRNA caused by repressed splicing in absence of PPD domains. The PPD domains are therefore involved in regulating export but not splicing of these transcripts.

2.3. PTB associates with PPD1 and PPD2

We next investigated the physical association of PTB with the PPD1 and PPD2 domains by RNA immunoprecipitation with anti-PTB antibody. Pulldown of both CPS and CPS-PRE transcripts suggested their association with PTB (Fig. 3A). Deletion of PPD1 region from intron (CPSΔPPD1) obliterated association with PTB, implying PPD1 is a strong PTB binding site in intron. However removal of

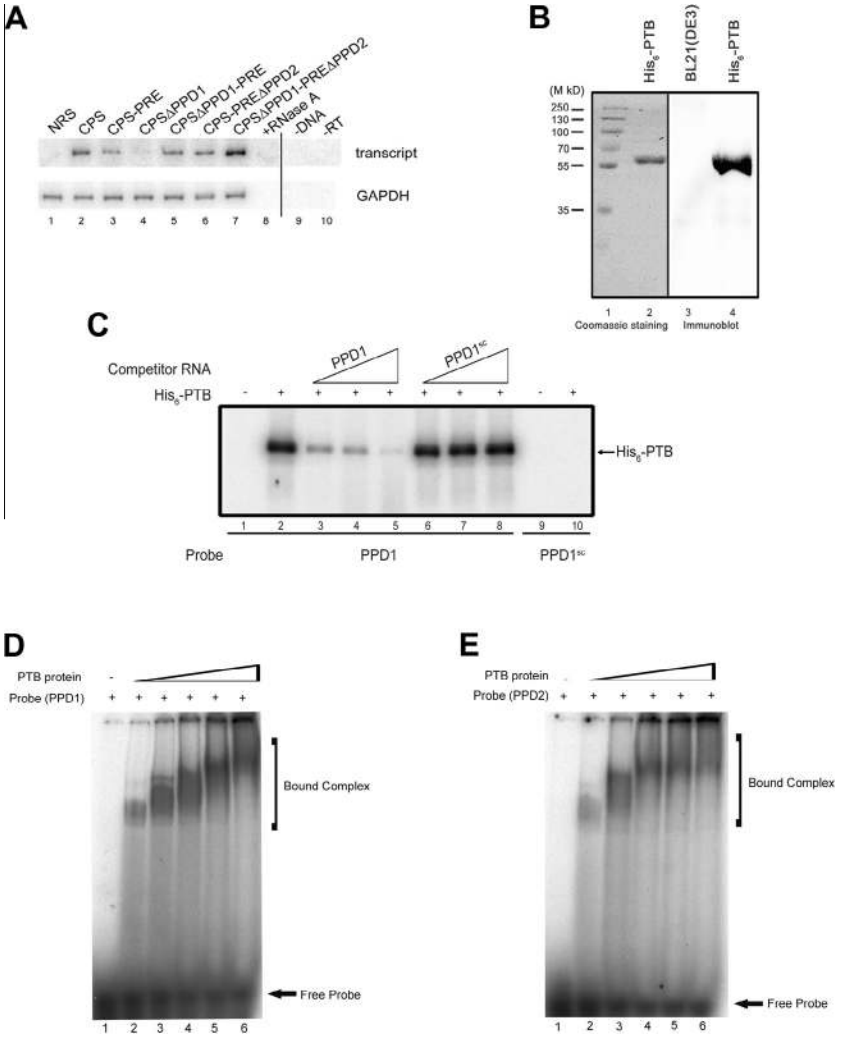

Fig. 3. PTB associates with PPD1 and PPD2 domains. (A) RNA immunoprecipitation with anti-PTB antibody followed by RT-PCR indicates association of PTB with PPD1 domain. Deletion of PPD1 abolished association between transcript and PTB (lane 4). PRE has multiple PTB interacting sites and PPD1 and PPD2 deletions did not alter PTB binding (lanes 5–7). Preimmune rabbit serum (NRS, lane 1) and RNase A (lane 8) establish PTB–RNA interaction. GAPDH transcripts control for RNA loading during pulldown. (B) Homogeneity of His_6-PTB used in the UV crosslinking and RNA-EMSA assays. (C) UV crosslinking shows stable association of PTB with pyrimidine bases in PPD1. Purines substituting for pyrimidines in PPD1 (PPD1^{sc}) could not compete for PTB binding. Other pyrimidine bases in PPD1^{sc} could not be crosslinked to PTB. (D and E) Both PPD1 and PPD2 sites can bind PTB and increasing PTB population shows accumulation of higher molecular weight complexes (lanes 2–6).

PPD2 site alone did not affect PTB binding to other pyrimidine rich domains present in CPSΔPPD1-PRE transcripts as PRE harbors several independent PTB binding sites [15]. UV crosslinking of PTB to PPD1 and its stable binding against a competitor where only the PPD1 pyrimidines were substituted by purines ($PPD1^{sc}$) confirmed the recognition of the PPD1 pyrimidines by PTB (Fig. 3C). Inability of $PPD1^{sc}$ to crosslink with PTB also indicates that PTB molecules were not in immediate contact with other neighboring pyrimidines in this oligomer. In RNA-EMSA, PPD1 and PPD2 formed diffuse complexes that increased in molecular weight with increase in PTB:RNA ratio (Fig. 3D and E). At least in PPD1 where only some specific pyrimidines were in direct contact with PTB, formation of higher molecular weight complexes possibly indicate PTB multimerization.

2.4. PTB overexpression represses cytosolic pre-mRNA levels

We next attempted a 'tethered function' assay for PTB. Constructs of full length PTB4 or its truncated RRM subdomains in tandem with MS2 coat protein were overexpressed in HuH7 cell lines. The expression of these different FLAG tagged proteins was confirmed by immunoblotting with anti-FLAG antibody (Fig. 4A). These PTB effectors were cotransfected with different reporter constructs to observe the effect of PTB overexpression on nuclear export. PPD domains in these reporter minigenes were either deleted or substituted by a single MS2 coat protein binding site. We observed that overexpression of full-length PTB4-MS2 imposed a considerable diminution in cytosolic pre-mRNA even in absence of MS2 coat protein binding site in the transcript (Fig. 4B). We think overwhelming repression by PTB4 expression and presence of only a single MS2 site masked any effect of via-MS2 interaction. Interestingly this squelching effect of PTB appeared to be exclusive to the presence of RRM4 domain. The PTB123L-MS2 protein retaining the other three RRMs except RRM4 did not show any effect.

We next explored the effects of deletions of PPD1 and PPD2 domains or PTB4-MS2 overexpression on splicing. Overexpression of PTB repressed splicing significantly in all minigene transcripts but deletions of PPD1 and PPD2 sites did not have any effect (Fig. 4C). Therefore in a PTB overloaded scenario repression of splicing led to nuclear accumulation of minigene pre-mRNA without any concomitant increase in cytosolic level. However PTB overexpression did not change nuclear and cytosolic profile of spliced GAPDH transcripts (data not shown). The export repression by PTB overexpression raise the following possibilities: (i) accumulated nuclear minigene pre-mRNA titrated specific nuclear export factors, or (ii) PTB overload removed a specific export factor from the minigene pre-mRNA, or (iii) synthesis of some important export factors itself was compromised due to PTB-dependent aberrant processing of their pre-mRNAs.

2.5. PPD mediated inhibition and β mediated stimulation dictate steady state level of pre-mRNA export

We next tried to address the relationship between the PPD domains and the role of PRE in nuclear export. Our focus was on the β subelement [27], a putative positive export regulatory region within PRE (Fig. 5A). We observed that deletion of β from the constructs uniformly abrogated pre-mRNA nuclear export. Therefore in our pre-mRNA minigene with endogenous HBV intron we recapitulated export promoting function in the β subelement of PRE [28]. However addition of PRE in the 3′ exon almost halved its nuclear export if compared with CPS pre-mRNA alone. This is concordant with observations that PRE as a whole did not promote export of HBV pgRNA [13]. We reasoned that PRE harbors both positive and negative elements for nuclear export and outcome of PRE will depend on the availability of cognate *cis* acting elements in the intron and *trans* acting factors in the nucleus (Fig. 5B). Positioning of PRE in the 3′ exon downstream of endogenous intron allowed the inhibitory network to suppress the positive effect of β. We suggest that both PPD1 and PPD2 sites on the minigene pre-mRNA allow PTB to aggregate on them to repress export.

Our finding about the role of PTB in retention of unspliced pre-mRNA transcript is intriguing. Association of *Xenopus* homolog of PTB as part of a multiprotein complex to Vg1 mRNAs is important for its cytoplasmic localization [29]. More importantly PTB strongly stimulated export of an unspliced heterologous transcript in a PRE

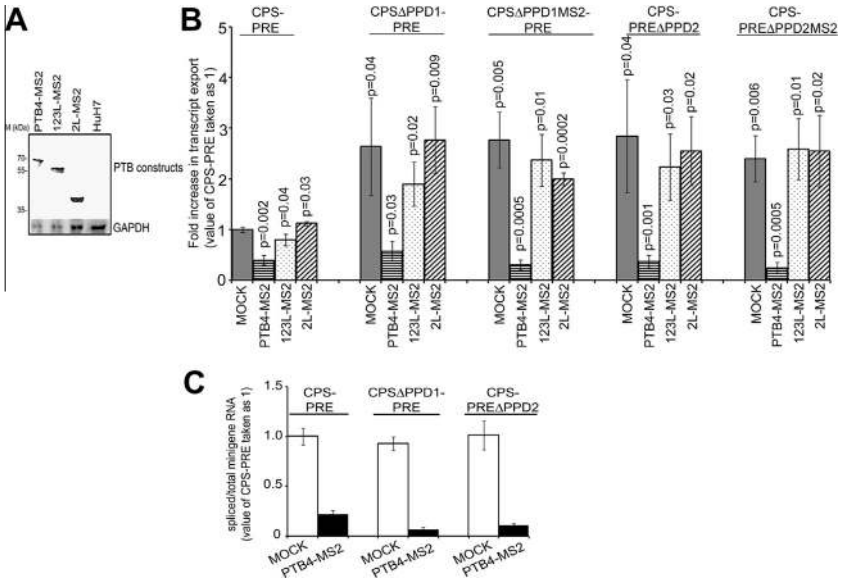

Fig. 4. Effect of PTB overexpression on export of minigene transcripts. (A) Anti-FLAG immunoblot shows expression profile of FLAG-tagged PTB and its derivatives in HuH7 cells. (B) Overexpression of PTB4-MS2 drastically reduced nuclear export of minigene pre-mRNA even without cognate MS2 coat protein binding site. CPSΔPPD1MS2-PRE and CPSPREΔPPD2MS2 have MS2 coat protein binding regions in PPD1 and PPD2 sites. RRM4-less PTB effectors were ineffective for this squelching. Experiments were carried out as described in Methods and Fig. 2. (C) Deletion of PPD1 and PPD2 domains was inconsequential toward splicing but PTB4-MS2 overexpression severely decreased splicing in all constructs. Splicing efficiency was calculated after direct measurement of spliced and unspliced species present in both nucleus and cytosol. The total transcript level was similar in all the cases.

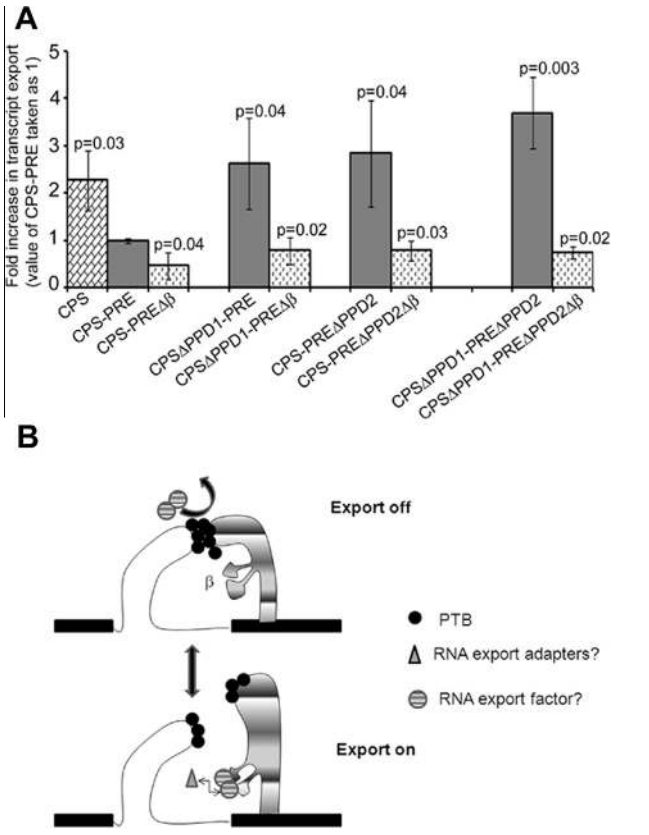

Fig. 5. PPD sites inhibit export stimulation from β region in PRE. (A) Deletion of β subelement (Δβ) of PRE uniformly repressed pre-mRNA export. PRE has an overall inhibitory effect when put 3′ to the endogenous intron. ΔPPD constructs allowed removal of PTB suppression on β activity resulting in higher export but this stimulation was lost on β removal. Experiments and calculations were done as described earlier. (B) Model depicting PTB as a mediator of nuclear export. Binding of PTB to PPD1 and PPD2 domains interferes with the interaction of β subelement with RNA export factors. Association between PTB molecules occludes β by bridging the PPD1 and PPD2 sites and establishes a equilibrium of pre-mRNA export.

dependent manner [15]. Inhibition of transcription of PTB by MyD88 downregulated PRE mediated stimulation of export of pre-S2/S RNAs [30]. However these reporter constructs contained PRE within a heterologous intron.

Importance of cooperative association between PTB molecules bound on both sides of skipped exons is already established in splicing [20]. PTB typically interacts with more than one polypyrimidine domains through its four RRMs, of which the RRM4 seems to be critical for splicing repression in $GABA_A$ receptor γ2 pre-mRNA [31]. We find it interesting that in our minigene system also, deletion of RRM4 domain from overexpressed PTB4-MS2 molecules obliterated PTB driven export repression. Secondly we also showed that both PPD1 in intron and PPD2 in exon were necessary for inhibition of export and perturbation of any of these two sites removed inhibition.

Results from our assay suggested that β region in PRE is a regulated export enhancer as presence of multiple pyrimidine domains across intron–exon junction blocked its activity. We suspect that in previous studies of PRE in the context of cDNA or intronless mRNAs [9,10], PTB binding domains were put outside the context of intron–exon junction thereby allowing the positive export regulatory element in PRE to work unhindered and stimulate export. On the other hand, PTB bound to HBV intronic sites may have interacted with other PTB molecules bound to polypyrimidine stretches in PRE and squelched export stimulation. Our findings suggest PTB as a possible metazoan candidate for nuclear retention of pre-mRNA. We also understand that this information can be used as a tool to interfere with HBV replication in infected hepatocytes.

3. Materials and methods

3.1. Minigene constructs

The CPS-PRE construct has been described [23]. All other constructs with changes in different regions of either CPS or PRE were made in pcDNA 3.1(+) using similar strategies. A single MS2 coat-protein binding site was synthesized by oligonucleotide annealing. The oligonucleotides used for different experiments are described in Supplementary Table 1.

3.2. Real-time RT-PCR

For real-time PCR analysis, 2 μg of extracted RNA was reverse-transcribed with RevertAid M-MuLV Reverse transcriptase (Fermentas Inc.) in a total volume of 20 μl. One microlitre of this reaction was amplified in StepOnePlus™ Real-Time PCR System (Applied Biosystems) using DyNAmo ColorFlash SYBR Green/ROX qPCR Master Mix(2×) (Thermo) and 0.3 μM specific primers. To ascertain purity of amplified PCR products, melting curve analysis was done after completion of PCR cycles. For validation, cDNAs were prepared from 10-fold serial dilutions of in vitro transcribed target RNAs and amplified alongside the test samples. Standard curves of C_t vs. log[RNA in ng] were drawn to ascertain the amplification efficiencies were comparable between the amplicons. The plot of ΔC_t (C_t of target – C_t of reference) against log[RNA in ng] had a slope of 0.092 allowing relative quantification by comparative threshold method ($2^{-\Delta\Delta C_t}$). Export of target transcripts in each sample was calculated against an exclusively nuclear transcript (U6) as reference. Normalization with cytosolic U6 level rectified any error arising out of variation in sample preparations. Fold difference between cytosolic and nuclear levels for the target transcript (extent of export) in any particular biological replicate is given by $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t$ (cytosol) – ΔC_t (nucleus), ΔC_t (cytosol) = $C_{t,target} - C_{t,U6}$, ΔC_t (nucleus) = $C_{t,target} - C_{t,U6}$. The obtained value for each construct was normalized against CPS-PRE for comparative analysis. Data shown here represent mean of at least three independent transfections with error bar representing ± standard deviation. Student's *t*-test was performed to obtain the *P*-value.

For calculation of splicing efficiencies, absolute quantities of unspliced and spliced RNA in each sample were obtained from the respective standard curves of C_t vs. log[RNA in ng] drawn with known amounts of in vitro transcribed target RNA. Specific primers used for detection of spliced RNA are already described. Similar quantitation of GAPDH mRNA level was done for normalization of minigene RNA level in cytosol.

See Supplementary methods for additional information.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.10.005.

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