Final Report

Project Title: Polypyrimidine tract binding protein (PTB): functions and its interaction with hepatitis B virus post-transcriptional regulation in living cells

By Asst. Prof. Nattanan T-Thienprasert

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Abstract

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Project Period: June 2015 – June 2016

Abstract: Hepatitis B virus (HBV) infection is a major cause of hepatocellular carcinoma and liver cirrhosis worldwide. Although HBV vaccination can successfully prevent new infections, safe and effective antiviral drugs are not available for 380 million people that are chronically infected with HBV. To develop novel antiviral drugs, a better understanding of the regulation of HBV gene expression is crucial. Previously, *in vitro* studies have shown that the HBV post-transcriptional regulatory element (HBV PRE) binds to endogenous polypyrimidine tract binding protein (PTB). However, the roles of PTB in the HBV PRE functions are still arguable. This project therefore aimed to investigate functions and interaction between HBV PRE and PTB in living cells. The exogenous PTB fused with yellow fluorescent protein derivative was successful constructed namely fPTBVenus. It maintained endogenous PTB's characteristic including same localization and ability to shuttle between the nucleus and the cytoplasm of the cell. Based on Trimolecular fluorescent complementation (TriFC), PTB was observed to interact with the full-length PRE in living cells. However, the core of PTB binding site will have to be further investigated. By co-transfecting luciferase reporter constructs with a plasmid expressing small interference RNA against PTB (psiPTB) in the presence and absence of HBV PRE, the luciferase assay revealed that the transfection of siPTB had no effect on the luciferase activity in the absence of PRE. Interestingly, addition of 100 ng of psiPTB could significantly increase luciferase activity in the presence of HBV PRE in both nuclear export and splicing reporter systems. However, a greater addition of siPTB (more than 200 ng) did not affect the luciferase activity in both reporter systems suggesting that other *trans* proteins may act to compensate with the loss of PTB. In contrast, overexpression of exogenous PTB (200 ng) had no effect on the expression of luciferase activity. Consequently, the results in this study indicated that the interaction between PTB and PRE plays important roles in the nuclear export and splicing mechanism of HBV mRNAs.

Keywords : Hepatitis B virus, PRE, PTB, nuclear export, splicing mechanism

Executive summary

Hepatitis B virus genome

Hepatitis B virus (HBV) is a DNA virus in *Hepadnaviridae* family (Ganem, 1996) Its genome is partially double stranded circular DNA of 3.2 kb that contains four primary overlapping open reading frames (ORFs) namely core (C), polymerase (P), surface (S) and X (Seeger and Mason, 2000) (Figure 1). HBV genome is reported to have five major transcripts, which are initiated at different sites by four major promoters: C, pre S1, preS2/S and X. There are two sets of C transcripts depending on the initiation sites of the C promoter. The longer C transcript (pcRNA) is initiated from the precore region and encodes the hepatitis B e antigen (HBeAg) whereas the shorter C transcript (pgRNA) is initiated from the core region and encodes the nucleocapsid protein (HBcAg) and the P protein. The preS1and preS2/S transcripts encode three different viral surface envelope proteins (HBsAg), which are large surface protein (preS1), middle surface protein (preS2) and small surface protein (S) respectively (Huan and Siddiqui, 1993). The X transcript encodes X proteins (HBxAg) that is reported to have multiple functions including DNA repair, transcriptional activation and inhibition of protein degradation (Bouchard and Schneider, 2004; Hu et al., 2006; Zhang et al., 2001). Notably, these major HBV transcripts are synthesised in the same direction by the host RNA polymerase II and are terminated at a single polyadenylation [poly (A)] site (Ganem, 1996) (Figure 1). Moreover, they are reported as the unspliced mRNAs and their nuclear export have been reported to be mediated by a *cis*-element known as HBV post-transcriptional regulatory element (HBV PRE)

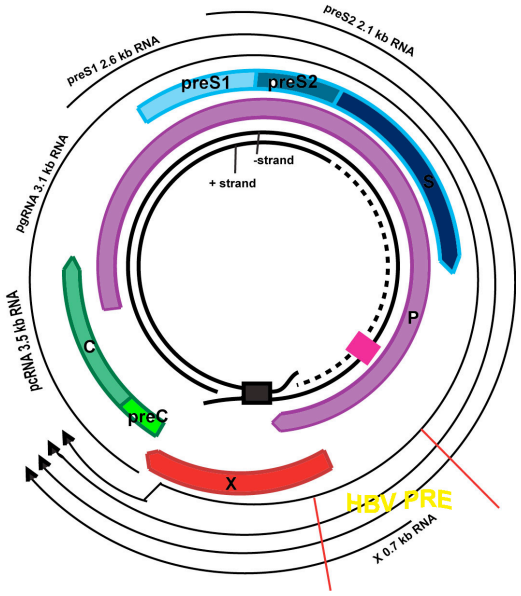

Figure 1: Schematic diagram of HBV genome. Both of negative and discontinuous positive strands DNA are shown in the centre circles. Broad arrows indicate the major viral ORFs. The outer thinner arrows around the genome represent the major viral transcripts. The boxes represent position of the enhancers I and II. The HBV post-transcriptional regulatory element (HBV PRE) region is indicated between two lines (nucleotide 1151-1684). (Adopted from Panjaworayan, 2007)

HBV post-transcriptional regulation (HBV PRE)

The HBV PRE is a conserved RNA element about 500 bases long found in all HBV transcripts. It has been reported as an important RNA export element for unspliced HBV mRNAs (Donello et al., 1996; Huang and Liang, 1993; Huang and Yen, 1995) and found to be involved in other regulation of HBV RNAs such as RNA splicing (Heise et al., 2006) and RNA stability (Ehlers et al., 2004). Recently, the computational analysis and deletion assay suggested that the HBV PRE is likely to contain multiple regulatory elements that may have different functions during the HBV lifecycle (Panjaworayan et al., 2010). Within HBV PRE, several regulatory elements have been reported including a human La binding site (Heise, Guidotti, and Chisari, 1999; Horke et al., 2002), stem-loop structures, HBV SLα and HBV SLβ (Smith et al., 1998), a *cis*-acting splicing regulatory element (SRE-1) (Heise et al., 2006), the binding sites (PRE III) of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and polypyrimidine tract binding protein (PTB) (Li et al., 2009; Zang et al., 1998; Zang et al., 2001; Zang and Yen, 1999) (Figure 2). However, the RNA-protein interaction between HBV PRE and PTB has not yet been confirmed in living cells. Moreover, the binding sites of HBV PRE on PTB have not been reported.

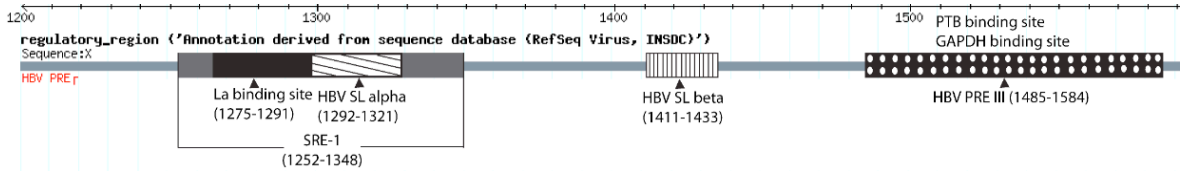

Figure 2: Graphic picture of HBV PRE with annotation of previously reported elements. (Modified from Panjaworayan *et al.*, 2010)

Polypyrimidine tract binding protein (PTB)

The PTB is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) family. It is 57 kDa protein that contains four consensus RNA recognition motifs (RRMs) (Ghetti et al., 1992). By using functional and structural analysis, Oh *et al* (1998) reported that RRM1 and RRM2 are required for PTB oligomerization and other protein-protein interactions (Oh et al., 1998) whereas Pérez *et al*. (1997) reported that RRM3 and RRM4 are important for specific and efficient RNA binding activity (Perez, McAfee, and Patton, 1997) PTB has been implicated in many processes of gene expression such as a negative regulator of pre-mRNA splicing (Lou et al., 1999; Valcarcel and Gebauer, 1997), RNA polyadenylation (Lou et al., 1999; Moreira et al., 1998) and RNA localization (Cote et al., 1999). Moreover, PTB has been reported to interact with internal ribosomal entry site (IRES) element of hepatitis A virus (HAV) and enhance cap-independent translation (Yi, Schultz, and Lemon, 2000). PTB is also indicated to bind with IRES of hepatitis C virus (HCV), but its role on the HCV replication is

unclear (Brocard et al., 2007; Chang and Luo, 2006). In addition, PTB has been demonstrated to mediate the export of HBV unspliced mRNA from the nucleus (Zang et al., 2001). However, PTB is well known as the splicing repressor by interfering with splice site definition (Amir-Ahmady et al., 2005; Carstens, Wagner, and Garcia-Blanco, 2000; Spellman et al., 2005). Therefore, the later experiment by Heise *et al*. (2006) was performed to investigate whether binding of PTB associated splicing factor (PSF) to HBV PRE affects splicing of HBV pgRNA or not. The results with regard to Heise *et al*. (2006) revealed that overexpression of the PSF could stimulate HBV pgRNA splicing. Heise *et al*. (2006) therefore speculated that PSF could compete polyrimidine tract with PTB resulting in loss of PTB function as the splicing repressor (Heise et al., 2006). Taken together, all previous results suggest that PTB plays important roles in the regulation of HBV gene expression, but the roles of PTB in the function of HBV PRE is unclear. Moreover, the detailed mechanism of how PTB acts in PRE function is still unknown.

Objective

1. To investigate the RNA-protein interaction between the HBV PRE with the PTB using Trimolecular Fluorescent Complementation" (TriFC) assay in living cells.

2. To identify roles of PTB in the function of HBV PRE by overexpression exogenous PTB and also knockdown the endogenous PTB in the presence and absence of HBV PRE

Research methodology

Plasmid preparation and validation

Two different plasmids expressing siRNAs were given from Dr. Chris Brown, Department of Biochemistry, Otago University, namely psiPTB1 (targeted on PTB mRNA) and pSiNegative (insertion of random human sequence). The double restriction digestion using *BamH*I and *Hind*III were performed. Next, the digested plasmids were analysed using gel electrophoresis.

Sequences of the full-length PTB cDNA and a deletion series of PTB sequences were amplified from a cDNA clone of PTBP1, namely pOTB7 (Open Biosystems) using PWO DNA polymerase enzyme (Roche) with specific primers: PTB1F (5'-gcgcgcGCTAGCGTGCCATGGACGG CATTGTCCC-3') and fPTBR (5'-gcgcgcCTCGAGGATGGTGGACTTGGAGAAGGAGACCCGC-3'). The PCR reaction mix was set up following manufacture's instruction from Roche Applied Science. The PCR programme was carried out using 'hot start' combined with 'touch down' PCR programme: 95°C for 5 min; 80°C for 30 sec; 30 cycles of 94°C for 30 sec, 87-67°C for 30 sec, 72°C for 30 sec; 5 cycles of 94°C for 30 sec, 67°C for 30 sec and 72°C for 2 min. PCR products were purified using QIAquick PCR purification kit (Qiagen). The full length PTB (fPTB) was digested with *Nhe*I (#R0131S) and *Xho*I (#R0146S) restriction enzymes at 37°C for 16 h. To deactivate restriction enzymes, the cut fPTB cDNA sequence underwent heat inactivation at 65°C for 20 minutes. The cut fPTB was inserted into the cut vector, pIMP1Venus (gifted from Brown laboratory, University of Otago) at *Nhe*I and *Xho*I sites by ligation. Plasmid construction was carried out with heat shock transformation (Section 2.6.8) and plasmid preparation using a commercial plasmid kit (Section 2.6.10). After validation of the plasmid sequence, the correct plasmid was designated as pfPTBVenus.

Two different plasmids expressing luciferase gene were utilized namely pBasic/fPRE, and pBasic without Intron (pBasic(-IN)) (Panjaworayan *et al*., 2010). These plasmids were constructed by using pGL3- MS2 site/Basic reporter. The pBasic/fPRE was constructed by inserting PRE sequence at the *Xho*I and *Nhe*I restriction enzyme site of pGL3-MS2 site/Basic. On the other hand, the pBasic(-IN) was constructed without PRE sequence inserted. To verify these plasmids, fast digestion of the restriction enzymes, plasmids and buffer were mixed together by following table 2.6. Then, the mixture was incubated at 37°C for 30 minutes then the enzyme mechanism was stopped by heating at 80°C for 5 minutes. At last the mixture was put into agarose gel.

In addition, TriFC plasmids were generously provided by Dr. Chris Brown (Department

of Biochemistry, Otago University) (Rackham and Brown, 2004). They were validated by DNA sequencing (Macrogen).

Cell culture

Two different cell lines were used in this study. They were human liver cancer cell (HepG2), human liver cell (HuH-7) and human cervical cancer cell (HeLa).

All cell lines were cultures in 75 cm^2 sterile tissue culture flasks (Greiner Bio-One) at 37°C with 5% CO2 in DMEM supplemented (Invitrogen) with 10% heat inactivated FBS (10% v/v) (Invitrogen), 1% L-glutamine (Invitrogen). For hepatocellular cell line, 1% (v/v) of non-essential amino acids (Invitrogen) was also included in the culture media. Cells were regularly passaged to maintain exponential growth. Basic cell culture techniques were carried out according to 'Current Protocols in Cell Biology' (Bonifacino, 1998).

Seeding cells and transfection

For luciferase activity, cells were seeded on 24-well plates (Greiner Bio-One) with a cell density approximately 1× 104 cells/mL in each well. Cells were incubated in an incubator at 37°C with 5% CO2 for 24 h prior to transfection. At this stage, cells would reach ~70% confluence. Transfection was then performed using the cationic lipid transfection reagent, FuGENE6 (Roche). The ratio between FuGENE6 (μL) and DNA (μg) was 3:1. FuGENE6 was mixed with serum free media to a total volume 100 μL and incubated for 5 minutes at room temperature. The FuGENE6-media mixture was then dropped into each sterile microcentrifuge tube containing 195 ng of reporter plasmid construct and 5 ng of phRL-SV40 (Renilla luciferase expression plasmid), unless otherwise indicated. A DNA transformation standard plasmid, pUC18 was also used to top up the total amount of DNA if required. The DNA-FuGENE6-media mixture was incubated for 15 minutes at room temperature and then added dropwise to the cells. In all experiments, cells were transfected at least in triplicate.

For experiments with confocal microscopy, cells were seeded on poly-D-lysine coated 35 mm tissue culture dishes with number 0 cover slip bottoms (MatTek) with cell density approximately 2 × 104 cells/mL in each dish. Cells were incubated in an incubator at 37°C with 5% CO2 for 24 h prior to transfection. FuGENE6 was used for transfection. The ratio between FuGENE6 (μL) and DNA (μg) was 3:1. FuGENE6 was mixed with serum free media to a total volume 100 μL. The FuGENE6-media mixture was then dropped into DNA plasmids expression fluorescent proteins. For the immunofluorescence study, cells were transfected with 200 ng of the plasmid expressing PTB fusion protein (fPTBVenus). For the

TriFC study, plasmids for expression of RNA reporters were transfected at 4× the molarity of fusion protein expression plasmids. Cells were transfected with the DNA-FuGENE6 mix after the mixture had been incubated at room temperature for 15 minutes. Forty-six h post-transfection, which is two h before acquiring the cell images with a confocal microscope, the growth medium was changed to fresh medium in each dish.

Luciferase activity

HepG2 were seeded in 24-wells plate approximately 1 x 105 and grown for 24 hours, and then co-transfected and incubated for 48 hours. Secondly cells lines were lysed by using PLB. Next, 50 µL of LAR II (luciferase assay substrate mixed with luciferase assay buffer II which can keep in -70°C for a year) was added into lysates then measured by using luminometer. After that, 1x of stop & Glo® was added 50 µL per well and measured again.

Confocal microscopy analysis

Two h after the replacement of growth medium (48 h after transfection), images of live cells were acquired using a confocal microscope. For detection of Venus fluorescent protein (VenusFP), both of the confocal microscopes were set to use laser lines at 514 nm in conjunction with a 530 nm longpass emission. For cells expressing the VenusFP and CFP, 514 nm and 458 nm lines of laser were used to excite them respectively. A 530 nm longpass and a 475-525 nm bandpass emission filters were used to detect the photons emitted from the VenusFP and CFP respectively. Samples were scanned in plane mode with an average of four linear scans for the final images. Images are single confocal slices with pinhole diameter of 579 µm. Detector gain was set to 812, and amplifier gain was set to 0.00 V.

Result and Discussion

Localisation and movement of the made exogenous PTB in living cells

The PTB fusion protein was tested to ensure that the fusion of Venus to the C-terminus of the PTB protein did not interfere with its localization within the cells. Thus, the pfPTBVenus (200 ng) was transiently transfected into two different cell lines, HuH-7 and HeLa cells. In particular, HeLa cell was also included in this experiment because the HeLa cell has been reported to form a perinucleolar compartment (PNC) within which PTB is found to localize (Ghetti et al., 1992; Huang et al., 1997). Forty- eight h post-transfection, images of cells were acquired using a confocal microscope.

As a result, cell images strongly indicated that the newly made PTB fusion protein was localized extensively in the nucleus of cell lines used in this study (Figure 1). These results were consistent with the observation reported earlier for native PTB and for the fusion protein containing GFP at the N-terminus of PTB (Huang et al., 2004; Kamath et al., 2001). Moreover, localization of PTB within the PNC was clearly demonstrated in the HeLa cell (Figure 1A) as previously reported by the immunofluorescence studies (Huang et al., 1997).

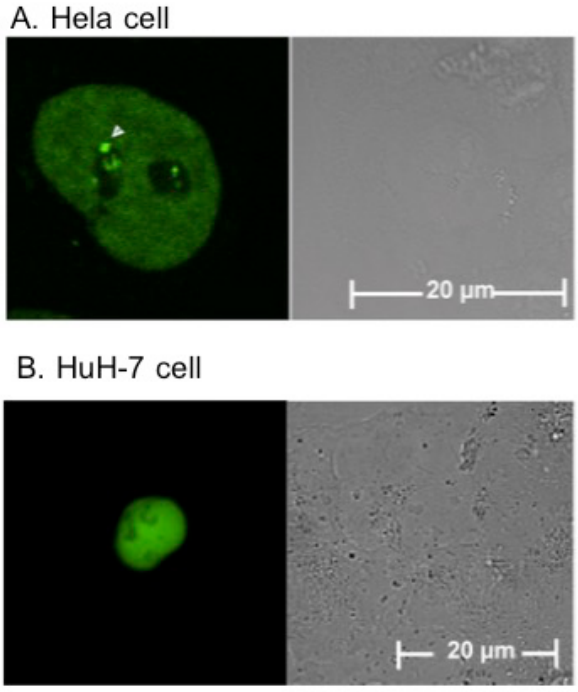

Figure 1: Localization of PTB fusion protein in living cells. (A) Localization of the PTB fusion protein in HeLa cells. (B) Localization of the PTB fusion protein in HuH-7 cells. The arrow indicates the PNC structure that is visualized in HeLa cells. Scale bar of each cell image is indicated. Images display phase contrast. The left panel views Venus signal. The right panel shows the phase contrast.

Next, FRAP study was performed to analyze movement of the PTB fusion protein between two compartments (nucleus and cytoplasm). COS-7 cells were then transiently transfected with 200 ng of the pfPTBVenus. A whole area of cell nucleus was then photobleached with 514 nm laser pulse. A series of images was acquired at 1-second interval.

After photobleach, there was no observation of PTB-Venus within the nucleus (1 second) (Figure 2). The fluorescence recovery of PTB-Venus was gradually observed in the nucleus after 2 seconds. The maximal recovery was complete after 22 seconds. However, the level of fluorescent recovery was not as high in fluorescent intensity as the prebleached cell. This is due to a decay of the fluorescent protein over time, especially after a very high laser pulse from the photobleaching. Subsequently, the results from this experiment indicated dynamic movement of the new fusion PTB protein from the cytoplasm to the nucleus.

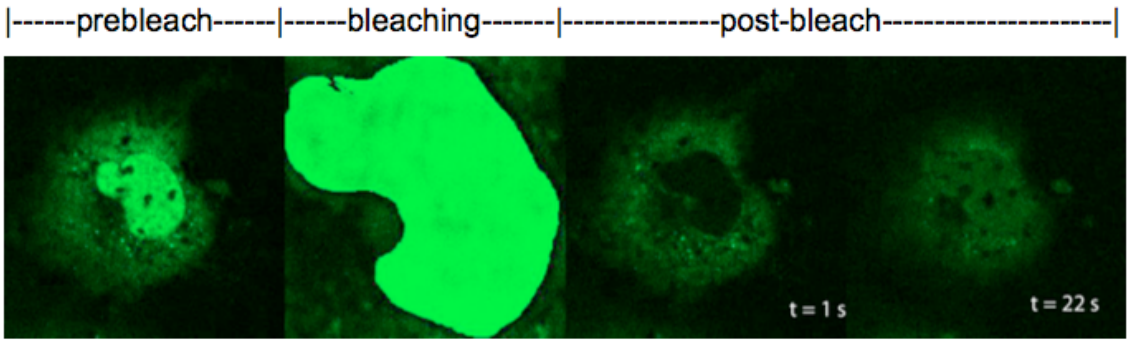

Figure 2: FRAP study demonstrated fluorescent recovery of the PTB fusion protein in live cells. Selected cell images indicating cells before and after photobleaching. COS-7 cells were transfected with 200 ng of the fPTBVenus construct. Forty-eight h post-transfection, cells were subjected to FRAP. The cell nucleus was bleached by a 514 nm laser pulse indicated by the enlarged imaged of nucleus during the bleaching step. A series of cell images were acquired at 1- second intervals. Time (seconds) is indicated.

Interaction between PTB and HBV PRE in living cell using TriFC technique

The TriFC assay has been developed to allow visualization of RNA-protein interaction in living mammalian cells. This assay extends the utility of tagging proteins with GFP derivatives to identify their cellular locations. The tag in the TriFC system is split in half: one half is fused to the target or 'prey' RNA binding protein while the other is tethered to a specific 'bait' RNA (Figure 3A). Thus, when the two halves are brought together, fluorescence is produced, which indicates where the RNA-protein interaction is actually occurring (Figure 3B).

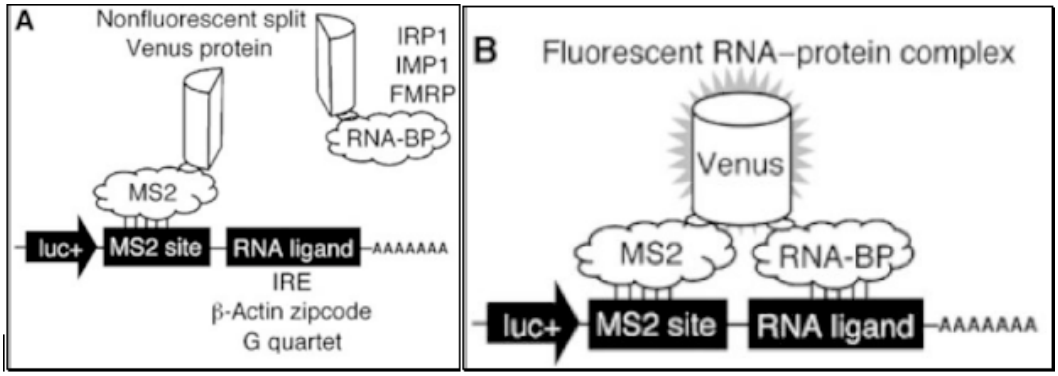

Figure 3: A trimolecular fluorescence complementation (TriFC). (A): A portion of the Venus fluorescent protein that is tethered to a specific bait RNA (bacteriophage MS2 coat protein-RNA interaction). The complementing portion of Venus is fused to the target or prey RNA binding protein. (B): The RNA-protein interaction brings two portions of Venus into close proximity to form a fluorescent product. For example, the TriFC assay has successfully detected interactions between IRP1-IRE, IMP1-β actin zipcode and FMRP- G quartet in living cells (Adopted from Rackham and Brown, 2004).

The TriFC experiment was aimed to investigate the interaction between fPRE and fPTB, thus cells were transiently transfected with the TriFC plasmids: (i) 60 ng of MS2- coat protein-RNA construct (pMS2Venus 1-154), (ii) 60 ng of binding protein construct (pfPTBVenusC) and (iii) 240 ng of RNA-ligand binding construct [pPRE (-IN)]. The ratio of plasmid expressing RNA reporter construct to the fusion protein expression plasmids was 4:1 as suggested in the publication (Rackham and Brown, 2004). As a result, the TriFC confirmed the interaction between PTB and PRE in the living cell (Figure 4).

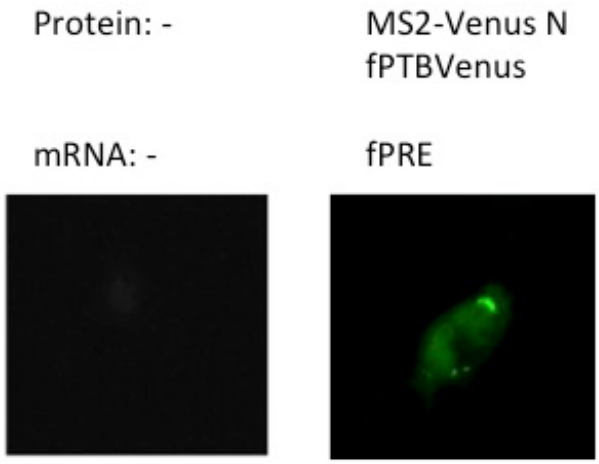

Figure 4: Interaction between PTB and HBV PRE using TriFC assay in COS-7 cell.

Functional study of PTB in HBV PRE function using luciferase activity

Several functions of PTB are involved in RNA metabolism, for example regulation of RNA splicing, RNA localization and RNA stability. Moreover, previously in vitro studies have shown that PTB can interact with HBV PRE (Zang *et al.,* 2001). However, the roles of endogenous PTB in HBV PRE functions are still arguable. As PRE have been reported to function in nuclear export and splicing of HBV mRNA, this project was then aim to investigate whether PTB plays role in HBV PRE function or not. The effect of knocking own PTB would be investigated using luciferase assay based on the specific luciferase reporter systems, nuclear export (pBasic(-In)) and splicing (pSpliceLuc) (Panjaworyan *et al*., 2010; Visootsat *et al*., 2015). To identify the PTB function, the HepG2 cells were co-transfected (Table 2.9 and 2.10) with various plasmids and extracted protein using PLB buffer. The lysates were added with luciferase substrates and then measured luciferase activity by using luminometer. Finally, the Firefly luciferase activity was normalized with Renilla luciferase.

From Figure 5, the mock condition (No.1), which was cells without transfection of any plasmids, had no luciferase activity confirming that non- transfected cells could not express luciferase protein. When cells were transfected with pBasic(-In) and psiNeg, the luciferase activity could be detected indicating that transfection was successful and it was used as the control. Then, the luciferase activity with knockdown PTB but without the presence of HBV PRE indicated that the luciferase activity was insignificantly decreased from the control. Thus, the effects of knockdown PTB did not affect the luciferase activity in the absence of HBV PRE. Next, the condition 3 was set up to see the effect of HBV PRE on the luciferase activity in the normal level of endogenous PTB and the result revealed that HBV PRE could strongly increase the luciferase activity. The condition 4-6 were set up for the purpose of studying effects of knockdown endogenous PTB in the presence of HBV PRE. By knocking down PTB with only 100 ng of psiPTB, the level of luciferase activity increased significantly ($p < 0.05$) when compared to the condition 3. This result may imply that PTB plays its role as the inhibitor of nuclear export. Surprisingly, increasing amount of psiPTB to 200 and 400 ng had no effect on the luciferase activity when compared to the condition 3. The result seemed to suggest that knocking down of PTB in a lower amount could increase luciferase activity in the presence of HBV PRE. PTB has not been reported clearly on the function involved in the nuclear export of mRNAs. Nevertheless, it has been well studied and known that the low level of PTB can trigger autoregulation of regulated unproductive splicing and translation (RUST). Moreover, its action can be compensated by the nPTB that is a paralog of PTB (David et al., 2000). To understand roles of PTB in nuclear export of HBV mRNAs, more experiments are required.

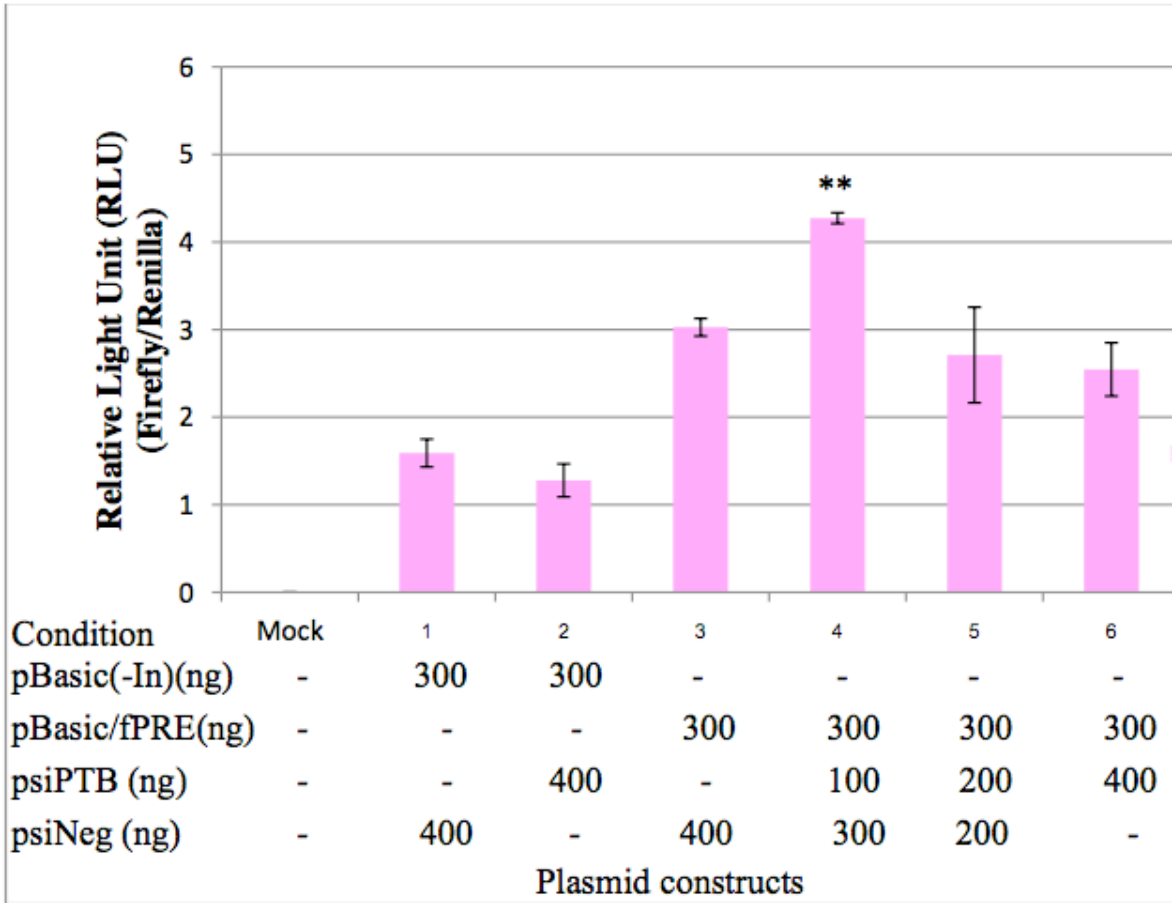

Figure 5: Effects of PTB knockdown on luciferase activities in the presence of HBV PRE by using luciferase report construct for the nuclear export system. * represents $p < 0.05$, ** represents $p < 0.01$, when compared to cells transfected with pBasic/fPRE but without psiPTB (Condition No.3).

Next, the experiment was set up to investigate roles of PTB-HBV PRE in a splicing mechanism using the pSpliceLUC reporter system (Panjaworayan *et al.*, 2010; Visootsat *et al.*, 2015). Cells were transfected with different plasmids as indicated in Figure 6. In this system, the presence of HBV PRE could also increase the luciferase activity (Figure 6, condition 3). Interestingly, addition of 100 ng of psiPTB could significantly increase the luciferase activity (Figure 6, condition 4) while the higher concentration of psiPTB (200 and 400 ng) had no effect on the luciferase activity (Figure 6, condition 5 and 6).

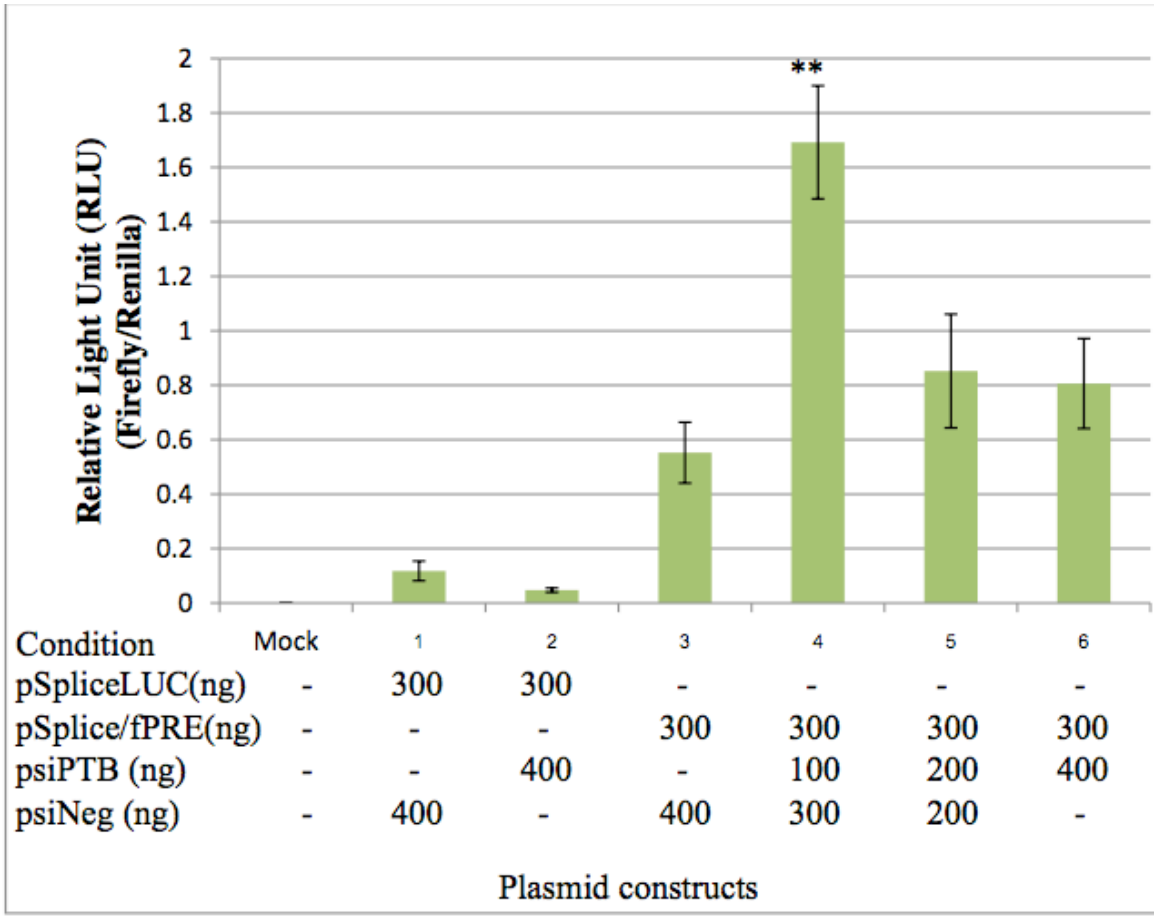

Figure 6: Effects of PTB knockdown on luciferase activities in the presence of PTB by using luciferase report construct for splicing system. *represents $p < 0.05$, ** represents $p < 0.01$.

Therefore, lower level of PTB could aid inhibition of splicing mechanism leading to a higher level of luciferase activity in the presence of HBV PRE ($p < 0.01$) (Figure 6, condition 4). However, when the concentration of PTB was very low, it was likely that the lost of PTB action was compensated or coupled by another mechanism. For example, the alternative splicing of eukaryotic mRNAs have been reported to work closely between PTB and the splicing suppressing function of SRp30c (Paradis *et al,,* 2007). However, it has been unclear whether SRp30c involved in any viral gene regulation.

Conclusion

1. The study successfully develop exogenous PTB that acted as the endogenous PTB i.e localization and movement patterns
2. By performing TriFC assay, PTB was observed to interact with PRE in living cells.
3. The study found that addition of 100 ng of siPTB could enhance luciferase activity in both nuclear export and splicing mechanism in the presence of HBV PRE. However, addition of higher amount of psiPTB (200 and 400 ng) had no effect on the luciferase activity.
4. More experiments are therefore required to understand the actions of PTB in HBV PRE functions. Nevertheless, the findings of this project demonstrated that the interaction of PTB and PRE plays important roles in the nuclear export and splicing mechanism of transcripts.

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