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# Role of Hepatitis B virus X protein (HBx) in cell adhesion and cytoskeletal reorganization : implications on virus replication 

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Role of Hepatitis B Virus X protein (HBx) in Cell Adhesion and Cytoskeletal Reorganization - Implications on Virus Replication

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SCHOOL OF BIOLOGICAL SCIENCES

# Role of Hepatitis B Virus X Protein (HBx) in Cell Adhesion and Cytoskeletal Reorganization <br> - Implications on Virus Replication 

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School of Biological Sciences

A thesis submitted to the Nanyang Technological University in fulfilment of the requirement for the degree of

Doctor of Philosophy

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## ABBREVIATIONS

| AKT | Protein kinase B |
| :---: | :---: |
| APS | Ammonium persulfate |
| ATCC | American type culture collection |
| B-PER | Bacterial - protein extraction reagent |
| bp | Basepair |
| cDNA | Complementary deoxyribonucleic acid |
| CREB | Cyclic-AMP responsive element binding |
| CRIB | Cdc42 / Rac1 interactive binding |
| DAPI | 4', 6-Diamidino-2-phenylindole |
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |
| ECM | Extracellular Matrix |
| EDTA | Ethylenediaminetetra-acetic acid |
| EGF | Epidermal growth factor |
| FBS | Fetal bovine serum |
| GFP | Green fluorescent protein |
| Grb2 | Growth factor receptor bound protein 2 |
| eGFP | Enhanced green fluorescent protein |
| HBV | Hepatitis B virus |
| HBsAg | Hepatitis B Virus Surface Antigen |


| HBeAg | Hepatitis B Virus E Antigen |
| :---: | :---: |
| HBcAg | Hepatitis B Virus Core antigen |
| HBx | Hepatitis B virus X protein |
| HCC | Hepatocellular carcinoma |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| IAA | Iodoacetamide |
| IPTG | Isopropyl- $\beta$-D-thiogalactopyranoside |
| IEF | Isoelectric focusing |
| JNK | c-JUN amino terminal kinase |
| Kb | Kilo base pair |
| kDa | Kilodalton |
| LB | Luria Bertani |
| LC | Liver Cirrhosis |
| MEM | Minimal Essential Medium Eagle |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| mRNA | Messenger ribonucleic acid |
| MW | Molecular weight |
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PI-3K | Phosphatidylinositol 3-kinase |
| PVDF | polyvinylidene difluoride |


| RFLP | Restriction fragment length polymorphism |
| :--- | :--- |
| R.T. | Room temperature $\left(\sim 25^{\circ} \mathrm{C}\right)$ |
| RT-PCR | Reverse transcription - polymerase chain |
| RT RT-PCR | reaction |
|  | Real time - reverse transcription polymerase |
| SDS | chain reaction |
| SDS-PAGE | Sodium dodecyl sulfate |
| SH2 | Sodium dodecyl sulfate - polyacrylamide electrophoresis |
| SH3 | Src-homology 2 |
| STAT | Src-homology 3 |
| TAE | Signal transducers and activators of |
| TGF- $\beta$ | transcription proteins |
| TEMED | Tris, acetic acid, and EDTA buffer |
| 2DE | Transforming growth factor- $\beta$ |

## Publications

(1) Tuan Lin Tan and Wei Ning Chen. (2005) A proteomics analysis of cellular proteins associated with HBV genotype-specific HBX: potential in identification of early diagnostic markers for HCC. J. Clin. Virol. 33: 293-298.
(2) Qi Chun Toh, Tuan Lin Tan, Wei Qiang Teo, Chin Yee Ho, Subhajeet Parida, Wei Ning Chen. (2005) Identification of Cellular Membrane Proteins Interacting with Hepatitis B Surface Antigen using Yeast Split-Ubiquitin System. Int. J. Med. Sci. 2(3): 114-117.
(3) Tuan Lin Tan, Zhiqin Feng, Yi Wei Lu, Vincent Chan, Wei Ning Chen. (2006) Adhesion contact kinetics of HepG2 cells during Hepatitis B virus replication: Involvement of SH3-binding motif in HBX. Biochim. Biophys. Acta - Mol. Basis Dis. 1762: 755-766.
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(8) Yi Wei Lu, Tuan Lin Tan, Jianhua Zhang, Wei Ning Chen. (2007) Apoptosis induced in different HBV genotypes: role of the HBSP. Virol. J. 4: 117.
(9) Tuan Lin Tan, Ning Fang, Tuan Ling Neo, Pritpal Singh, Jianhua Zhang, Ruijie Zhou, Cheng-Gee Koh, Vincent Chan, Seng Gee Lim and Wei Ning Chen. (2007) Rac1 GTPase Is Activated by Hepatitis B Virus Replication - Involvement of HBX. Biochim. Biophys. Acta - Mol. Cell Res. (In Press)

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Poster Presentation
Session 8 Regulatory Proteins and Carcinogenesis (Poster - 115)
"HBX Interacts with Cytoskeletal Proteins through SH3 Binding"
Tuan Lin Tan, Zhiqin Feng, Vincent Chan, Wei Ning Chen.
(2) 2007 International Meeting - The Molecular Biology of Hepatitis B Viruses September 16-20 th 2007 Rome, Italy

Oral Presentation (By A/P William Chen Wei Ning)
Session 8 HCC and Carcinogenesis (Oral - 145)
"Activation of small GTP-binding Protein Rac1 by HBV replication"
Tuan Lin Tan, Ning Fang, Cheng Gee Koh, Vincent Chan, Wei Ning Chen*.
*Presented by A/P William Chen Wei Ning

## Summary

HBV genotypes have been linked to differential liver disease outcomes. For genotypes that are predominant in Asia, HBV genotype $B$ has been shown to lead to hepatocellular carcinoma while the genotype C has been associated with more invasive liver disease. Using 2-Dimensional Electrophoresis, we identified differences in proteome expression profiles in cells transfected with constructs expressing distinct genotypes of the smallest HBV protein, HBx, which has been linked to oncogenic development.

In addition to the analysis of global proteome profile, we have also identified a short proline rich region within the HBX containing PXXP motifs reminiscent of a SH3 domain binding motif, a key link in the transmission of cellular signaling. It has been shown that the HIV viral protein Nef interacts with cellular PAK-1 through its PXXP motif resulting in increased viral replication. The HCV non-structural viral protein NS5A has also been shown to interact with Grb2 through the same PXXP and SH3 domain interaction, resulting in perturbation of the mitogenic signaling. For HBV, HBx might interact with c-Src directly which might potentially lead to HCC.

Through the use of bacterial-expressed proline-rich region from HBx , we identified multiple interacting partners containing SH3 domains on a protein array. Many of these serve important roles in cell cytoskeleton maintenance and cellular signaling. In particular, we showed that Vinexin $\beta$ (a binding partner of vinculin for the formation of focal adhesion complex) interacted with HBx through the PXXP motif. This provided
molecular understanding on the delay in cell adhesion associated with either the HBV replication or HBx expression, as we observed concurrently. Significantly the delay in adhesion was restored in cells transfected with HBx construct containing proline to alanine mutations within the PXXP motif. Our results suggested that HBx was directly involved, via the SH 3 binding domain, in the cytoskeletal reorganization leading to alterations in the cell adhesion process.

To determine the functional significance on HBV replication, structural analysis by Atomic Force Microscopy and fluorescent microscopy was carried out in cells supporting HBV replication and those expressing Rho p21 proteins which are key players in cellular cytoskeletal reorganization. We showed that cells expressing the constitutively activated Rac1 displayed similar morphology to those supporting HBV replication. Moreover, we showed that HBV activated Rac1 specifically compared to Cdc42 through activated-GTPase binding assay to PAK1 Cdc42/Rac1 interactive binding (CRIB) domain. Significantly, we showed that both activated Rac1 and Cdc42 led to an upregulation of HBV viral replication as evidenced by increased viral intermediates through quantitative Real Time-RT-PCR. Transient transfections of HBx have been shown to result in similar phenotypic morphology as rHBV transfected cells and it was also shown to be potentially interacting with $\beta$ PIX, a RhoGEF responsible for Rac1 activation.

Taken together, our results provided direct evidence of interaction between HBx and cytoskeletal modifying proteins. The interaction of HBX with the p21-RhoGTPases may be functionally important for sustaining HBV replication as well cytoskeletal
trafficking of viral components. Such interactions leading to modifications of normal cytoskeletal functions may explain the involvement of HBx in the development of hepatocellular carcinoma. The new virus-host interactions sites identified in our project may in turn be used as novel target for antiviral actions to reduce HBV-related liver diseases.

## Chapter 1 Introduction

### 1.1 Hepatitis B Virus

Hepatitis B Virus (HBV), a para-retrovirus which affects many people worldwide belongs to the family of Hepadnaviridae. Upon initial infection, HBV rarely causes acute fulminant hepatitis which results in severe liver damage of varying severity dependent on host immune response. Most of the asymptomatic HBV infected individuals end up as chronic carriers. More then 350 million people worldwide currently have been infected with HBV and have become asymptomatic carriers (Yen, 1996). As such, they spread the disease to a further extent unknowingly. A large proportion of these HBV carriers eventually develop severe liver diseases such as liver cirrhosis which more often then not leads to hepatocellular carcinoma, a leading killer in the world (Ganem and Schneider, 2001). In general, HBV spreads through similar routes as Human Acquired Immunodeficiency Virus (HIV), involving the direct contact of bodily fluids such as semen and blood from carriers to victims. Most importantly, the virus can be spread easily from mother to child during childbirth and hence it is important to understand and have good therapeutic measures against this viral infection.

Progress in understanding HBV have been slow due to many issues such as (1) inherent naturally-occurring mutations (leading to various serotypes and genotypes found to date), (2) lack of suitable animal models for understanding the disease causing effect of HBV (Tree-shrew is commonly used for this purpose but maintenance cost as well as aggressiveness of animal makes it difficult to work with), (3) lack of recognition of its importance and severity, due to the discovery of a recombinant vaccine since 1984 (McAleer et. al., 1984). Although a vaccine is in place, this vaccine do not offer lifetime
protection and requires booster vaccinations to maintain protective immunity against HBV infection. In addition, vaccines have no effect on chronic HBV carriers and the current drugs interventions (Lamivudine and $\alpha$ - interferon) are not working very well in prevention of disease progression due to emergence of drug resistance strains.


Figure 1.1: HBV viral particles under electron microscope. This picture shows the 3 different types of viral particles, namely (1) Dane particles (2) 20 nm spheres and (3) 20 nm filamentous particle. Adapted from:
http://www.stanford.edu/group/virus/hep adna/2004tansilvis/Patient \% 20Ed.htm

In the blood of patients suffering from persistant HBV infection, at least three types of particles can be found (Figure 1.1). The most important of these particles is what is known as the Dane particles with an approximate size of $42-47 \mathrm{~nm}$. In particular, the Dane particles are double shelled particles and is the infectious virion encompassing the HBV viral genome (Dane et. al., 1970). The other two particles found through electron microscopy are (a) 20 nm spheres as well as (b) different lengths of 20 nm diameter filaments. The three particles possess a similar characteristic in that all of them have the Hepatitis B surface antigen (HBsAg). It is widely believed that the non-viral genome containing viral particles serve as deterrent forces to cellular immune response allowing the Dane particle to have increased chance of establishing infection in susceptible liver cells. Besides the HBsAg in the outer shell, the Dane particles contain another shell consisting of the nucleocapsid of approximately $25-27 \mathrm{~nm}$ in diameter, essentially made
up of HBV viral core protein. The core protein can be detected as a 21 kDa protein, designated HBcAg and have been used as indicative factor for persistant HBV infection besides HBsAg (Summers et. al., 1975).

### 1.1.1 HBV Genome

HBV has a compact genome of $\sim 3.2 \mathrm{~kb}$ of partially double-stranded DNA and replicates by reverse transcription through an RNA intermediate, the pregenomic RNA of $\sim 3.5 \mathrm{~Kb}$. The viral genome is packaged inside the nucleocapsid and viral envelop which forms the double-shelled Dane particles mentioned earlier. This compact genome possesses various promoter and enhancer elements and overlapping coding regions for the various viral proteins and hence requires complex regulation for proper viral propagation (Seeger and Mason, 2000).


Figure 1.2: Arrangement of the overlapping viral genomes of HBV adw2 serotype. Numbering of viral sequences was designated from the conserved EcoRI restriction site. Pregenome ( $\sim 3.5 \mathrm{~Kb}$ ) shown as the outer ring, is one of the most important viral mRNA reverse-transcribed for the replication. (adapted from Arbuthnot et. al., 2000)

The HBV viral DNA encodes for 4 overlapping viral genes encoding viral proteins: Core (HBcAg), X (HBx), Surface (HBsAg) and Polymerase as shown in the different colored bars (Figure 1.2). One or more promoters regulate each of these genes and they are in turn, globally controlled by one or more viral enhancer elements (Enh1 and Enh2) themselves. Enh1 and Enh2 are located upstream of the core promoter. In particular, Surface gene consists of three in frame ATG start sites coding for the Large (L), medium (M) and small (S) surface antigen (as indicated by PreS1, PreS2 and S respectively) and possess the same stop site encoded at the end of the $S$ gene. Essentially, core and surface viral proteins are involved in the packaging of the viral genome while HBx's role still remains ambiguous, but have been shown to have linkage to HCC (Ganem and Schneider, 2001).

The viral polymerase ORF spans the largest of the viral genome overlaps all the other 3 viral ORFs (Figure 1.2) and starts its translation from an internal initiation codon using pgRNA as the template. It consists mainly of the amino and carboxyl terminal separated by a spacer region. The amino terminal is termed the terminal protein (TP) and is responsible for both the priming of minus strand HBV DNA and also important for the pgRNA packaging (Seeger and Mason, 2000). On the other hand, the carboxy terminal encodes the reverse transcriptase (RT) with RNaseH activity. The viral polymerase is the least abundantly expressed probably due to the fact that it binds to the pregenome at the 5' end upon translation, preventing further gene expression.

As indicated on Figure 1.2, DR1 and DR2 are direct repeats of short 11 nucleotides sequences which are important for the priming of the synthesis of the two asymmetrical DNA strands, namely the minus (-) and plus (+) strands. Essentially, minus strand starts its 5' end at DR1 while the plus strand, shown within the inner ring, begins with DR2 (Seeger et. al., 1984, Will et. al., 1987).

### 1.1.2 HBV Replication

HBV replication takes place in the host cell and consists of various critical steps including: (1) Formation of cccDNA, (2) Viral gene expression and viral protein synthesis and (3) Reverse transcription and assembly (Seeger and Mason, 2000). After viral entry, conversion of the HBV relaxed circular viral genome into covalently closed circular DNA (cccDNA) in the host cell nucleus, is a critical limiting step indicative of successful infection by the virus. However, the mechanisms, probably involving DNA repair at the single strand region, removal of the attached viral polymerase and finally a ligation of the strands, remains unclear (Ganem and Schneider, 2001). In the nucleus, the cccDNA serves as the template for the transcription of the various viral mRNAs. The entire process of viral replication is schematized in Figure 1.3.

Viral mRNAs are transcribed from the cccDNA in host cell's nucleus. They are namely the longest pregenomic RNA ( 3.5 kb ) described earlier, and various other RNA with varying lengths of $2.4 \mathrm{~Kb}, 2.1 \mathrm{~Kb}$ and 0.7 Kb (Refer to Figure 1.2 external rings). The pregenomic RNA serves as the most important RNA template for both the core protein as well as the viral polymerase, each with different open reading frame (ORF).

Interestingly, core particles are expressed in excess of polymerase although they share the same viral mRNA template (Bartenschlager and Schaller, 1992, Crowther et. al., 1994). The intricate control for this differential expression remains unclear but could be due to the binding of the polymerase to its own mRNA upon expression, preventing further transcription and largely due to it being a downstream open reading frame. The binding of the polymerase to the viral pregenome is required for reverse transcription and packaging (Wang and Seeger, 1993). The production of more core particles with respect to polymerase by HBV viral replication is also due to the fact that more core particles are required during formation of the nucleocapsids encompassing the only one pregenome and polymerase.


Figure 1.3: HBV virus replication: After viral entry, key steps involve formation of cccDNA followed by viral gene expression and eventually viral protein production. Assembly and packaging of the viral genome into a nucleocapsid is followed by minus and plus strand synthesis. Eventually, the virion is transported out of the cell. (Adapted from Ganem and Prince, 2004)

The other shorter mRNAs code for the varying lengths of surface gene as well as the $X$ protein. As indicated in Figure 1.2, $S$ codes for the shortest definitive form of the $S$ domain (S). With extension of the PreS2 (M) and PreS1 + PreS2 (L), the surface genes expressed are termed medium (M) and Large (L) surface protein respectively. These envelop proteins all undergo glycosylation and are essentially type II transmembrane proteins found on the surface of the outer shell of the Dane particles (Dane et. al., 1970). Although vaccine developed against the smallest $S$ viral protein works effectively and can recognize both the $M$ and $L$ surface viral protein, presence of the longer $S$ proteins are believed to confer certain forms of resistance against anti-virals (Lambert et. al., 1990, 1991).

The shortest mRNA codes for the X protein which has been shown to be important for viral replication in animal viral infection (Chen et. al., 1993), but appears not to have any significant role in cell culture based viral replication (Zoulim et. al., 1994). X protein (HBx) appears to play various important roles in the cell after infection which are described in the later sections.

The final important step in HBV replication is the packaging of the genome and reverse transcription. The viral pregenomic RNA and polymerase are sequestered into viral core particles (Wang et. al., 1994), triggered by binding of viral reverse transcriptase to the unique N-terminal epsilon RNA stem-loop structure (at 5' end of pgRNA) (Pollack and Ganem, 1993). The viral reverse transcriptase acts as the protein primer for priming of the viral DNA synthesis from the pgRNA. After only 4 base
syntheses, the polymerase interestingly translocates to the other end of the pgRNA where the 4 bases bind complementary sequence. Hence, elongation occurs towards the N terminal of the pregenome forming what's depicted in Figure 1.2 as the $(-)$ minus strand. The RNaseH activity encoded at the C-terminal of the polymerase degrades the (+) plus strand leaving only approximately 17-18 bp at the amino-end of the plus-strand. This acts as primer for plus-strand synthesis. Another interesting fact is that the plus-strand remains varied in length and is rarely completed upon budding of the mature virion (Seeger and Mason, 2000), hence depicted as dotted lines in Figure 1.2.

### 1.2 HBV and Liver Diseases

Despite its small genome, HBV is known to be a major causative agent for a wide range of chronic liver diseases including hepatocellular carcinoma (HCC) and liver cirrhosis, affecting more than 350 million worldwide (Yen, 1996, Chen et al., 1999). HCC ranks as one of the deadliest diseases, resulting in approximately 1 million deaths per year and is the fifth most common cancer worldwide (Freeman et. al., 2002) Besides, HBV infection also results in fulminant hepatitis which occurs less frequently but often leads to premature death. Liver tissue damage is usually due to host immune response leading to targeting of the infected liver cells by host immune complexes. These damages vary in degree of severity and could potentially predispose the liver cells to hepatocellular carcinoma during inherent liver self recovery after scarring.

### 1.2.1 Higher Risk of HBV Carriers in Development of HCC

Patients infected(R)1 at their young age usually present less sever liver scarring and(R)1
 probably due to1 a1 certain1 degree of tolerance to $1 \mathrm{v}(\mathrm{R})$ iral1 antigen. 1 However, older infected $(R R) 1$ patients with more severe liver damage also1represents the more respon( R )sive1 group to1anti$v(R)$ irall treatment, 1 with higher chance of clearing the $v(R)$ irus due tol their immune respon( $R$ )se ( $\operatorname{Brook}(e \mathbb{R})$ all., 1989, Hoofnagle and( R)1DiBisceglie, 1997). 1

HBV carriers have1been1hown1to1have1a1 greater1risk1of more that a 100 fold increase in1isk1 of developing HCC (Abuthnot and(R)1Kew, 12001). Moreover, a correlation( R)1between HBV infection( R)1 and( R)1 incidence of HCC has been1 establihed( R)1 (Figure1 k. $A$,adnd( R)1 Kew1 1981). However, the und( R )erlying mechanisms remain1 tol be elucidated. Besides the pred( $R$ )isposition( $R$ ) 1 of cells to( $R) 1$ HCC during initial1 damage, it 1 has come to( $R) 1$ light that cer HBV $\mathrm{v}(\mathrm{R})$ iral1 protein1 such1 as HBx might either1 $\mathrm{d}(\mathrm{R})$ irectly1 cause cancer formation( R$) 1$ o pred( R )isposes cell to( R$) 1$ HCC due to( R$) 1$ carcinogen 1 expa\&ur20(T) H Zhu let. al., 2004, Wang et. al., 2004, Lee et. al., 2005, Zhang et. al., 2005). 1


### 1.2.2 Direct Carcinogenesis by HBV Infection

Early studies conducted showed a close correlation between HCC and chronic HBV infections (Sherlock et. al., 1970, Szmuness, 1978, Kew, 1981). It was found that HBsAg prevalence in HCC incidence can reach up to $85 \%$. There was also a high incidence of integration of HBV genome (usually random and partial) into chromosomal DNA of hepatocytes from HCC patients (Bréchot et. al., 1981, Shafritz and Kew, 1981). Therefore, the involvement of HBV predisposing liver cells to malignancy constitutes a major disease causative factor, together with those brought about by HCV infection or alcoholic abuse.

HCC development is a multistep process (Sugimara, 1992), including the host response to viral HBV. As mentioned earlier, HBV DNA integration with chromosomal DNA of malignant liver cells are common (Bréchot et. al., 1981). However, such integration process plays no role in viral replication itself. This process of integration is random and integration sites are highly variable. HBV viral proteins have been shown to activate transcription and the best studied examples are those of the X protein as well as the Large and Medium surface proteins (Chisari et. al., 1987, Meyer et. al., 1992). HBx have also been shown to bind to CREB (Maguire et. al., 1991) and activate MAP kinase and JAK/STAT pathways to be explained later.

Like other viruses, it is possible that HBV viral proteins interact directly with cellular proteins such as those of the cytoskeleton to facilitate the movement of progeny particles. This interaction may result in deregulation of the normal cellular architecture
hence, predisposing liver cells to malignancy. In addition, various signaling pathways attenuated by HBx might also result in pro-survival condition of the liver cells. Indeed, a direct role of HBV in liver carcinogenesis was supported by a recent finding (Zheng et. al., 2007).

### 1.2.3 Indirect Carcinogenesis by HBV Infection

Indirect hepatocarcinogenesis usually occurs with damage done to the liver prior to onset of HCC. For example, liver cirrhosis has been implicated in cancer as one of the causes leading to HCC development (Beasley et. al., 1981). After liver cells scarring, hepatocytes turnover increases which results in an equally increased risk of cumulated mutations in chromosomes. Such accumulated mutations potentially lead to malignancy. Concurrently, the increased cell division rate results in decreased time for repair of DNA damage before liver cells divide again. As a result, the accumulation of mutations might contribute to tumor development (Sugimara, 1992). Such hepatocytes become premalignant and are termed "initiated" hepatocytes. Initiated hepatocytes are shown to respond more fervently to carcinogenesis on exposure to carcinogen.

### 1.2.4 Need for Early Biomarkers

Due to the drastically shortened life expectancy after the onset of HCC and limited treatment options, its diagnosis has become increasingly important. The most frequently used HCC detection marker nowadays is $\alpha$-fetoprotein (AFP) (Tan et. al., 2003). However, these studies were conducted using end-stage HCC tissue and may not be useful in the context of early diagnosis/prognosis. Furthermore, $70 \%$ of HCC patients
have AFP levels lower then threshold concentration ( $500 \mathrm{ng} / \mathrm{ml}$ ) defined for HCC (Seow et. al., 2001). Recent studies have also indicated the variability of AFP with high concentration in chronic HBV carriers without HCC. Taken together, the identification of new markers for early diagnosis of HCC is much needed.

### 1.3 HBV Genotypes \& Disease Outcome

Like other viral reverse transcriptases which are devoid of proof-reading during reverse transcription, HBV has been shown to have $\sim 1.4-3.2 \times 10^{-5}$ mutations/ site per year). The rate of mutation in HBV was shown to be $10^{-2}$ fold lesser probably due to lower reverse transcriptase activity (Orito et. al., 1989. Henceforth, many serotypes, subtypes and genotypes have been evolved and eventually lead to differential disease outcome.

HBV strains have been categorized into different antigenic subtypes and later into nucleotide-divergence based genotypes (Kidd-Ljunggren et. al., 2002). Variant HBV genomes were first studied and segregated into 9 serotypes namely aywl, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq${ }^{+}$and adrq${ }^{-}$(Swenson et. al., 1991). A more distinct classification is first determined due to an intergroup difference of approximately $8 \%$ of genetic sequences, and is initially classified into 4 genotypes namely $\mathrm{A}, \mathrm{B}, \mathrm{C}$ and D by Okamoto et al. 1988. Subsequently, another 2 genotypes designated $\mathrm{E} \& \mathrm{~F}$, is defined by variability in the Surface genetic sequences (Norder et. al., 1992, 1994). Recently, another variant termed genotype G has been added based on its $11.8 \%$ difference from the previous 6 genotypes (Stuyver et. al., 2000).

HBV genotypes are found to be segregated worldwide in a rather conserved geographical distribution. Of importance, HBV genotypes B and C are found mainly in Asia, compared to genotype A, which is found mostly in Europe, USA and Central Africa. The other genotypes are rare and found in the Mediterranean areas, India, West Africa, South America while genotype H has been defined recently in France and USA. These differential localization have been summarized in Table 1.1 (Kao et. al., 2002, Schaefer, 2005). As such, differences can be observed in their overall nucleotide sequences as well as the comparative difference in viral polymerase length.

Table 1.1 Summary of the various genotypes and their worldwide distribution. Also, different genotypes of HBV show different genome length and viral polymerase length.

| Genotype | Subtype | Localization | Genome <br> Length <br> $(\boldsymbol{b p})$ | Length of <br> polymerase |
| :--- | :--- | :--- | :--- | :--- |
| A | $a d w 2$, ayw1 | North-Western Europe, <br> USA, Central Africa | 3221 | 845 aa |
| B | $a d w 2$, ayw1 | Taiwan, Japan, <br> Indonesia, China, <br> Vietnam | 3215 | 843 aa |
| C | adw2, adrq+, <br> adrq-, ayr | East Asia, Taiwan, <br> Korea, China, Japan, <br> Polynesia, Vietnam | 3215 | 843 aa |
| D | $a y w 2$, ayw3 | Mediterranean area, India | 3182 | 832 aa |
| E | $a y w 4$ | West Africa | 3212 | 842 aa |
| F | $a d w 4 q-$, <br> $a d w 2$, ayw4 | Central and South <br> America, Polynesia | 3215 | 843 aa |
| G | $a d w 2$ | France, USA | 3248 | 842 aa |

Earlier studies have pointed to a clinical link between HBV serotypes and vaccine escape mutants (Chen et. al., 2001a) and it has now been suggested that particular HBV genotypes may correlate with differences in clinical features of viral infection (Kao et. al.,
2000). For example, patients with genotype $C$ are more likely to have liver cirrhosis whereas those with genotype B are more likely to develop HCC (Kao et. al., 2002a, Chan et. al., 2003). Recently, patients infected with HBV genotype B have been found to follow a less aggressive route with a lower rate of liver cirrhosis (Fung and Lok, 2004). These patients also undergo HBeAg seroconversion at a younger age and exhibit less active liver disease compared to genotype C carriers. This has been further confirmed by Chan et. al., 2004, who have reported that patients infected with HBV genotype C are associated with persistent expression of HBeAg and that it is an independent factor for HCC development besides liver cirrhosis.

Besides the importance of the clinically more aggressive HBV of genotype C, Schaefer et. al., 2005 have highlighted the need to understand the other genotypes A, D, E , as this will aid in a more significant global understanding of how the differences between genotypes can result in such differential clinical presentations. In addition, these clinical studies could possibly be used in determination of treatment course in different patients (Schaefer et. al., 2005).

Therefore, clinical data have suggested that HBV genotypes might play differential role in disease progression. Given that most carriers are of genotype C in Asia, it is important to try to understand how it specifically leads to disease progression. Besides potential use as determinants of disease prognosis, HBV genotypes also show differential responses to anti-viral therapy. In particular, genotype B carriers have been shown to respond more effectively to interferon treatment compared to genotype C carriers in

Taiwan. It has also been suggested that this might be due to a higher proportion of genotype C carriers with basal core promoter (BCP) mutations (Kao et. al., 2002b). Contradictory results have however been shown for studies carried out on genotype A and D where it has been shown that genotype A carriers show good responses to interferon (Hou et. al., 2001). This has however not been observed in another study conducted in Japan (Kobayashi et. al., 2002). It is also possible that minute mutations can have diverse clinical outcomes even amongst the genotype themselves.

Hence, with genotypes B and C HBV prevalence in Asia where HBV carriers are endemic, it would be important to understand and elucidate the differences that genotypes confer to the virus to result in different disease outcome. These differences in nucleotide sequences between viral genotypes would eventually be translated into viral proteins with significant mutations at the functional level. Therefore, the study of the effects of viral proteins, such as HBx which has been implicated in many aspects of HBV-host interaction, of various genotypes should be crucial in our understanding of their effects on disease outcome.

### 1.4 Hepatitis B Virus X Protein (HBx)

HBx is a viral protein of the HBV that has been linked to the development of HCC. However, its direct role remains controversial despite various efforts to understand this elusive and yet seemingly promiscuous protein (Bouchard \& Schneider, 2004, Andrisani \& Barnabas, 1999, Murakami, 2001). It has been reported that HBx amino- and carboxyterminal regions contain helical domains (Colgrove et. al., 1989). In addition, it has been
shown that a 50 amino acid amino terminal deletion resulted in increased transcriptional activity and thus it has been postulated that HBx is a potential negative regulator which binds to cellular proteins and affects downstream function (Murakami et. al., 1994).

### 1.4.1 HBx Localization

Localization analysis has shown that HBx is located in the cytosol predominantly. Various studies have indicated a close localization to proteasome (Chen et. al., 2001b) as well as mitochondria (Takada et. al., 1999). HBx has been shown to bind via its Cterminal transactivation domain to PSMA1, a proteasome subunit. This binding is important to localize HBx to proteasomes at the peri-nuclear region where proteasome inhibitors play a role to prevent HBx degrading rapidly (Zhang et. al., 2000). Due to its overexpression, HBx might have been found to be in the cytoplasm although it could also be a nuclear protein (Henkler et. al., 2001). Hence, different localization of the HBx might be important in determining the role they play in the host cells.

### 1.4.2 HBx in Viral Replication

Contradictory results have been reported on the effects of HBx on HBV cell cycle. Although it has been shown in mice that HBx plays a role in upregulating viral replication, possibly by activating HBV viral core and surface gene expression (Xu et. al., 2002), it has also been shown HBV replication can occur without HBx (Blum et. al., 1992). On the other hand, HBx has been shown to play a transactivation role. Although it has not been shown to bind DNA directly, it may interact with other cellular proteins involved in the transcription process. It has been previously shown that HBx interacts
with various nuclear transcription factors such as TFIIB, TFIIH besides the TATA box binding protein (TBP). In addition, it has been shown that HBx interacts directly with the cyclic-AMP responsive element binding (CREB), hypothesized to be through a coiled coil motif in HBx to the b-zip CREB region. Such an interaction has resulted in an increased DNA binding capacity of the CREB protein (Maguire et. al., 1991).

### 1.4.3 HBx and Hepatocarcinogenesis

Albeit its short coding region, it has been widely studied for its role in hepatocarcinogenesis. HBx transgenic mice have been studied and shown by various reports to develop malignant transformation upon induction (Kim et al., 1991, Ullrich et. al., 1994). Likewise, It has been reported that $\mathrm{C} 57-\mathrm{TgN} \mathrm{X}$ transgenic mice are more susceptible to HCC upon introduction of carcinogen, Diethylnitrosamine (DEN), with the incidence of HCC development at about 3-fold higher compared to that of wild-type mice (Zhu et. al., 2004). Hence, it has been speculated that HBx may also play an indirect role by "sensitizing" hepatocytes towards HCC if it is not the only causative viral factor (Slagle et. al., 1996, Madden et. al., 2001).

### 1.4.4 HBx Interaction with p53

HBx has been shown to inhibit p53 (a tumor suppressor protein), and has been suggested to be part of process of early hepatocytes transformation leading to HCC. It has been shown by Ueda et. al., 1995 that HBx binds p53 which resulted in a decrease of p53 in the nucleus. This decrease in nuclear p53 concentration leads to the inhibition of hepatocytes apoptosis and deregulation of cell cycle control, which could play a role in
progression to HCC. It has also been shown recently that HBx binding to p53 results in the down-expression of PTEN (another tumor suppressor), the negative regulator of AKT (Protein Kinase B) and phosphatidylinositol 3' Kinase (PI-3K). PTEN has a p53 binding site in its promoter and decrease expression of PTEN due to decreased p53 will result in the de-repression of PI-3K by PTEN and activation of PI-3K downstream processes such as activation of AKT whose role includes cell growth and survival (Chung et. al., 2003).

### 1.4.5 HBx Activation of Various Signaling Transduction Cascades

In addition to $\mathrm{p} 53, \mathrm{HBx}$ has been shown to interact or bind to various X -responsive cis-elements of transcription such as CREB (Maguire et. al., 1991), NF-кB and AP-1. In addition, it has been shown to interact with various signaling transducing pathways such as Mitogen-activated protein kinase (MAPK) and Janus Kinase/Signal transducer and activators of transcription (JAK/STAT). As seen in Figure 1.5, HBx has been demonstrated to have a stimulatory effect on JAK/STAT signal transduction through activation of JAK phosphorylation. Also, HBx's activation of Src protein tyrosine kinases also has potential effects on the MAPK and JAK/STAT pathways (Arbuthnot et. al., 2000). Through these interactions, HBx may alter the normal signaling transducing pathways leading to malignant development of the hepatocytes.

HBx has been found to increase transcription of various proto-oncogenes such as cJun, N-Myc and c-Myc (Yen, 1996). In addition, it seems to play important role in the upregulation of transcription of various important transcription factors such as AP-1, NF$\kappa \mathrm{B}$ and CREB. As HBx is localized mainly in the cytoplasm (Chen et. al., 2001b), it can
potentially modify signaling transduction pathways, hence regulating their transactivating potential.


Figure 1.5: Effects of HBx on various signaling pathways such as MAPK, JAK / STAT and its potential role in hepatocarcinogenesis. (Adapted from Arbuthnot et. al., 2000)

HBx overexpression has resulted in an increased activity of protein kinase $C$, hence activating downstream transcription factors, AP-1 and NF-кB (Cross et. al., 1993, Haviv et. al., 1995). NF- kB is an important effector of HBx stimulation of various promoters of gene expression in hepatocytes. For this case, HBx has been shown to phosphorylate NF$\kappa \mathrm{B}$ inhibitors such as $\mathrm{I} \kappa \mathrm{B} \alpha$. This phosphorylation degrades the inhibitors, leading to tumor necrosis factor-alpha TNF $\alpha$ stimulation, eventually allowing translocation of NFкB into the nucleus (Weil et. al., 1999).

Various studies (Benn and Schneider, 1994, Henkler et. al., 1998) have shown that cytoplasmic HBx results in the activation of established Ras-Raf-MAPK signal
transduction cascade specifically (Doria et. al., 1995). Although no direct role has been established between HBx and the Ras/Raf/MAPK, it has been shown to activate Ras to the GTP-bound form. Recently, Klein et. al., 1999, have shown an activation of Src tyrosine kinase by HBx and potentially activates Ras pathway. This specific Src activation also plays key role in viral replication by increasing viral polymerase activation.

HBx has been shown to cause an upregulation of SAPK/JNK pathway (Diao et. al., 2001). Interestingly, phosphorylation of the postulated 14-3-3 protein binding domain of the HBx is required for the upregulation of SAPK activity. This eventually leads to a protection of the HBx transfected cells against apoptosis, leading to the formation of immortalized cells causing transformation of these hepatocytes. In addition, HBx has also been shown to activate phosphatidylinositol 3-kinase (PI-3K) leading to downstream AKT phosphorylation. AKT phosphorylation can potentially lead to anti-apoptotic effect by inducing transforming growth factor beta (TGF $\beta$ ) (Shih et. al., 2000). The mechanism of PI-3K activation remains unclear but could be through mediators e.g. cytokines such as IL-6 or epidermal growth factor (EGF) (Menzo et. al., 1993). Other indirect activation of AKT phosphorylation could be achieved through the Src/Ras-GTP or Jak1/STAT signaling pathways.

Another major signaling transduction pathway that HBx may potentially augment is that of the Jak-STAT signaling pathway (Lee and Yun, 1998). All the STAT kinases showed enhanced tyrosine phosphorylation upon introduction of exogenous HBx. It has
been hypothesized that the region 26-142 a.a. of HBx may bind to Jak1 specifically and directly. Hence, HBx binding might act as an adaptor molecule for bringing Jak1 close together resulting in auto-phosphorylation leading to downstream phosphorylation of STAT kinases. As summarized in Figure 1.5, these reported findings seems to point towards the pro-survival characteristics of HBx , which in turn suggest that HBx may have a significant role in the development of HCC. Interestingly, it has been shown recently that HBx integrated in host chromosome of HCC tissues has retained the ability to block p53-induced apoptosis. The authors have suggested that this might allow for selection for neoplastic hepatocytes in HBV carriers with integrated HBx hence contributing to hepatocarcinogenesis (Huo et. al., 2001). Interestingly, it has earlier been shown that such mutants displayed abolished pro-apoptotic effect of HBx , thus contributing to HCC development (Sirma et. al., 1999).

### 1.4.6 HBx and Apoptosis

While the above studies have indicated HBx as a pro-survival viral protein, many others have shown the apoptotic effects as a result of HBx expression in mammalian liver cells. HBx has been shown to display apoptotic effect on transient transfection in Huh7 cells (Kanda et. al., 2004). Similar phenomena of cell death was previously observed by Shintani et. al., 1999 by the usage of Cre/loxP recombination system to introduce HBx into two liver cell lines, HLF and HepG2. It has also been speculated that HBx causes apoptosis by sensitizing cells through the disruption of p53 function (Hsieh et. al., 2003). Another group has shown that HBx causes the loss of mitochondrial membrane potential leading to cell death (Shirakata and Koike, 2003). Recently, our lab has shown the
presence of a BH3-like domain within the HBx. Like other BH3 only pro-apoptotic proteins, HBx has been shown to cause apoptosis in HepG2 cells (Lu and Chen, 2005). These reports point to the ability of HBx to induce apoptosis. It is possible that HBx mediates either pro- or anti-apoptotic effects through its interactions with different cellular proteins and pathways.

### 1.4.7 HBx Functional Domains / Proline-Serine Rich Region

Various domains of the small HBx protein have been mapped out. As seen in Figure 1.6, the amino-terminal portion of the HBx corresponds to the regulatory domain while the carboxy-terminal encodes the transactivating domain, which has been the focus of intensive studies. In contrast, the proline-serine rich region from a.a. $30-46$ received the least attention although the region presented a large degree of mutation within HBx (Hwang et. al., 2003). In this thesis, we explore the potential of this particular region of HBx to serve as SH3-binding proline-rich motifs and demonstrate functional interaction with cellular cytoskeleton proteins as well as guanine nucleotide exchange factor. Significantly, recent clinical studies have indicated that a single nucleotide change within this region at a.a. 38 has been found in patients suffering from HBV-related HCC. The potential of using this mutation as a marker for predicting disease outcome in patients infected with HBV genotype C has also been explored (Muroyama et. al., 2006). Further understanding on the interaction of this region with cellular signaling may be important for the development of new therapeutic drugs / ligands for treatment of HBV carriers.


Figure 1.6 HBx structural domains. The multi-functional 154 a.a. HBx protein of the HBV possesses two main domains, Regulatory and Transactivating at the N terminal and C-terminal respectively. They are linked by a proline-serine rich (PSR) domain. BD - Basic domain, CPBD - Cellular protein binding domain. (Adapted from Sirma et. al., 1999)

### 1.5 Proteomics

The Proteome of a cell is a dynamic entity which changes with the different physiological states of the cell. Being the equivalent in nature of the transcriptome in DNA microarray, it aims to understand the end state production of specific proteins with response to the external stimuli. Proteomics has recently been harnessed for the understanding of the mechanisms of disease development such as cirrhosis and especially hepatocellular carcinoma (Liang et. al., 2002). Two-dimensional electrophoresis (2DE) is a method of choice for the separation of proteins and often forms the first screening protocol for proteomics. It has first been introduced by O'Farrell 1975 and J. Klose in the same year. This technique allows the resolution of 1000s of proteins and has since been refined to generate highly reproducible proteome maps by the advent of immobilized pH gradient (IPG) gel strips.

Various groups have recently made use of 2DE, with aim for biomarker discovery in HCC. Changes in expression levels of 21 proteins has been identified (Lim et. al., 2002),
of which some potential biomarkers such as sarcosine dehydrogenase, liver carboxylesterase and lamin B1 have been found to be upregulated in HCC tissues. On the other hand, Kim et. al., 2002 have compared proteome profiles between healthy and HCC tissues within individual patients, and found an increase in expression of protein disulfide isomerase A3 (previously reported to be increased in breast ductal carcinoma tissues as well as hypoxia-insulted glial cells). Other proteins of interest identified in the same study include HSP27, glycerol-3-phosphate dehydrogenase, cathepsin D, acyl-CoA dehydrogenase, catechol O-methyltransferase among others. They have been found to be down-regulated in most of the HCC tissues. In the same year, Park et. al. have reported a decrease in tissue-ferritin light chain (T-FLC) from HCC proteome. Coupled with RTPCR, it has been shown that the suppression of T-FLC is due to translational or posttranslational modifications. Levels of human aldehyde dehydrogenase isozymes (ALDH) have also been identified to be modified during HCC development (Park et. al., 2002)

It has been proven that $2-\mathrm{DE}$ is a powerful and reproducible method for identifying potential biomarkers as well as hastening drug discovery. However, most of the studies have been conducted on HCC tissues and results are often ambiguous due to the heterogenecity of transformed tissue samples. In addition, the aetiology of HCC is multifactorial and hence, it is not possible to directly establish the link between the identified proteins with their disease causing potency. Therefore, we propose a cellculture based assay to understand the early cellular response to HBV infection and proteins identified could then be further used to understand how HBV and HBX can contribute to hepatocarcinogenesis.

### 1.6 Src Homology-3 Domain and Proline-rich Motifs

In eukaryotic cells, Src homology-3 domains (SH3) represents as one of the most used modular domains found in many signaling proteins such as Src, Fyn, Lyn, Hck, Abl, Grb2, kinases, phosphatases, phospholipases and adaptor proteins. Spanning approximately 55-70 a.a., these non-catalytic segment displays specific interaction with proline-rich motifs. In other proteins, it has first been discovered in v-Src oncogene and more proteins containing the SH 3 modules have been found subsequently. They are especially found in conjunction with SH 2 and PH domain-containing proteins (Dalgarno et. al., 1997). Besides their role in signaling cascade, SH3 containing proteins also mediate interactions with cytoskeletal proteins. SH3 domains are also found in structural proteins (Stefan and Alan, 2000). Using phage display libraries, it has been shown that SH3 domains interact preferentially with proline rich motifs bearing the "PXXP" motif, where P codes for Proline while X codes for any other amino acids.

SH3 binding motif can be split into class I and II based on differences in the surrounding non-proline residues. Effectively, short regions with +XXPXXP and PXXPX+ are classified into class I and II respectively. Although SH3 domains bind proteins with PXXP motif, they exhibit certain amount of specificity. There are however some proteins such as Src , which binds peptides of both classes of proline rich motifs (Kay et. al., 2000).

SH3 domains bind targets with affinities of $\mathrm{K}_{d}$ (nanomolar to picomolar concentrations) between 1 to $200 \mu \mathrm{M}$ (Nguyen et. al., 1998) and is typically composed of
five $\beta$-strands uniquely organized into two sheets which are packed at right angles in a sandwich like conformation. Specifically, the more hydrophobic half of the sandwich constitutes the SH3 ligand binding surface (Cesareni et. al., 2002).

Proline-rich SH3 binding motifs have been found in many signaling proteins such as p21-activating kinases (PAK1), and even viral proteins. Of interest, Nef protein of HIV and NS5A from HCV, are both non-structural proteins that play a role in their respective viral replication. It has been shown that they possess proline-rich motifs which allow them to interact with host SH 3 domains containing cellular proteins. It is therefore likely that viral proteins can interact with cellular signaling pathway via SH3 domain to facilitate their replication but in the process resulting in downstream disease development.

### 1.7 Objectives of Research Project

The objective of this project is to identify and characterize cellular proteins associated with infection of specific HBV genotypes by proteomics approach, in an established cellbased HBV replication system which simulates early stages of HCC development. In addition, proteins secreted into culture medium will also be studied for their potential as early diagnostic HCC markers.

Experimentally, viral DNA will be extracted from serum samples and the genotypes will be determined by various techniques such as Polymerase Chain Reaction (Naito et. al., 2001) and/or Restriction Fragment Length Polymorphism (RFLP) (Mizokami et. al., 1999). Replicative genomes of the confirmed genotypes (B \& C) will be constructed as
described (Chen et. al., 2000a and 2000b), prior to the transfection into the nontransformed liver cell line, Chang Liver Cells (ATCC-CCL13) to mimic HBV replication in vivo. Total proteins will be extracted from the HBV replicating cells and twodimensional electrophoresis will be carried out to generate expression profiles of proteome between untransfected cells and those transfected with HBx or of genomes of specific genotype. Comparisons made on the proteomes generated by the transfection of the 2 specific replicative HBV genotypes ( B and C ) as well as their respective HBx will allow a more elaborate understanding of the early events in the development of HCC. The newly identified proteins should provide useful information on cellular signaling pathways due to early cellular response to HBV genotype-related infection. In addition, similar proteomic approach will be applied to culture medium from cells exposed to HBV genome or HBX of specific genotypes, which may provide useful information on new biomarkers for the early diagnosis of HCC.

During literature search and database mining, a proline-rich hyper variable domain was identified in HBx. Like other SH3 binding proteins, this domain may be potential ligands for SH3 domain-containing cellular proteins. In particular, HBx genotype B showed a typical PXXP motif, characteristic of a SH3 domain ligand while HBx of genotypes A and C contains non-canonical PXP or PXXXP SH3 binding motif. We applied biochemical, biophysical and microscopic analysis on the effect of this domain on HBV replication and the morphological changes in the HBx producing cells.

As exemplified in earlier section, HBx possess a proline-rich region. As a nonstructural viral protein implicated in HBV viral replication, it might potentially play similar role to Nef and NS5A in interaction with cellular proteins containing SH3 domain. Work on this was carried out in this thesis in the hope of getting more insight into how HBx might interfere with normal cellular signaling and result in downstream disease progression.

## Chapter 2 <br> Materials \& Methods

### 2.1 Hepatitis B Virus Clinical Samples

### 2.1.1 Multiple Sequence Alignment Analysis

Full length sequences of Hepatitis B virus for the various genotypes A to H (8 for each of the genotypes except genotypes $\mathrm{F}, \mathrm{G}$ and H due to limited amount of data submitted to Genbank) were obtained from Genbank, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/ENTREZ/nucleotide). They were aligned using VectorNTI suite's AlignX software and serve as a point of reference for primer design utilizing the known conserved regions of each genome. Between genotypes, there were approximately $8 \%$ differences in nucleotide sequences as previously described (Okamoto et. al., 1988). Also, there are differences, albeit minor (< $1 \%$ ) within each genotype themselves observed in the alignment results. It is important to note that besides nucleotide differences, different genotypes of HBV present different length as tabulated in Table 1.1. Significantly, HBV DNA of genotype A spans 3221 bp while genotypes B and C specifically spans 3215 bp due to naturally occurring 6 bp deletion, resulting in a 2 aa shorter polymerase in the latter 2 genotypes of HBV (Table 1.1).

### 2.1.2 Viral DNA Extraction from Clinical Serum Sample

Frozen serum samples were obtained from HBV-infected patients, from Singapore General Hospital (SGH) and National University of Singapore (NUH). A special thanks to Dr C J Oon and Dr S G Lim, respectively for kindly supplying the serum for our research. Hepatitis B viral DNA from Genotypes B and C specifically, were extracted using QIAamp Blood Mini Kit (QIAGEN) as per manufacturer's instructions.

Briefly, $200 \mu \mathrm{l}$ of serum were mixed with $20 \mu \mathrm{l}$ of QIAGEN proteinase K. $200 \mu \mathrm{l}$ of lysis buffer, buffer OL (proprietary to QIAGEN) were then added and mixed by pulse vortexing for 15 sec . The mixture were then incubated at $56{ }^{\circ} \mathrm{C}$ for 10 min prior to addition to addition of $200 \mu \mathrm{l}$ of analytical grade ethanol (96-100 \%). Thorough mixing was performed by pulse vortexing for an additional 15 sec prior to application onto QIAamp spin columns provided. HBV viral DNA were bound to the columns by centrifugation at $8,000 \mathrm{rpm}$ for 1 min using a microcentrifuge. Bound viral DNA were then first subjected to wash by $500 \mu \mathrm{l}$ of buffer AW1 (proprietary to QIAGEN) and centrifuged at $8,000 \mathrm{rpm}$ for 1 min . A second wash with $500 \mu \mathrm{l}$ buffer AW2 (proprietary) was then carried out by centrifugation at $14,000 \mathrm{rpm}$ for 3 min . Excess AW2 buffer were removed by further centrifugation at $14,000 \mathrm{rpm}$ for 1 min . HBV viral DNA were eluted with $50 \mu \mathrm{l}$ of elution buffer, buffer AE ( 10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0 ) and stored at $-20{ }^{\circ} \mathrm{C}$ prior to further use. Viral DNAs obtained serve as templates for viral genome and viral protein cloning

### 2.1.3 Confirmation of HBV Genotypes of Clinical Serum

Genotypes of the extracted HBV DNA were confirmed by a modified Restriction Fragment Length Polymorphism (RFLP) method as previously described (Mizokami et. al., 1999). First, the entire surface antigen gene, comprising the PreS1 and PreS2 region and the entire $S$ gene proper ( 2874 bp to 833 bp ), as observed in Figure 1.2 were amplified by primers P1 [5'-TCA CCA TAT TCT TGG GAA CAA-3'] and P2 [5'-GTT TTG TTA GGG TTT AAA TG-3']. This was followed by sequencing using primer P3 [5'-TCC TGC TGG TGG CTC CAG TTC-3'] which is prior to the position of nucleotide

155, essentially the Start codon of the smallest surface antigen gene (S). Sequencing results were aligned in silico with VectorNTI suite's AlignX software with database sequences. Sequencing results were analyzed in silico instead of physical enzymatic digestion and agarose gel run as exemplified in Figure 3.1 (B).

### 2.1.4 Cloning of Viral Genomes/Genes in pCDNA3.1+

The replicative competent genomes for Hepatitis B virus comprises of approximately 3.5 Kb fragment (referred to as 1.1X HBV genome as HBV genome is $\sim 3.2 \mathrm{~Kb}$ ) cloned into a mammalian expression vector such as pCDNA3.1+ (Invitrogen Inc., USA). Essentially, the enhancer II (EnhII) situated within nucleotides position 1600 - 1900 are present in wild type partial double stranded region. Therefore, a two step cloning strategy, essentially the PCR of 2 fragments flanked by a 1600 to 1900 bp fragment (containing the critical replication elements of HBV - Direct repeat 1 (DR 1), Direct repeat 2 (DR2) and Enhancer II (EnhII)), is utilized in the generation of replicative genomes of HBV genotypes B and C , into a mammalian expression vector, pCDNA3.1+.

Primers P4 [5'-GTG ACG CGT CAC GTC GCA TGG AGA CCA CCG-3'] and P5 [5’-AGA ACG CGT TCT AGA CTC TGT GGT ATT GTG AGG-3'] were used to generate the first fragment and is flanked by MluI sites (underlined), comprising of an inner HBV intrinsic XbaI site at the 3' end (bold). The second fragment stretching from 248bp to 1900bp was generated using P6 [5'-AGT CTA GAC TCG TGG TGG ACT TCT CTC-3'] and P7 [5'-GCT CTA GAG CCC TAA AGC CAC CCA AGG C-3'], flanked by XbaI sites (bold) for integration into the first fragment. For ease of cloning as
well as safety reasons (minimal handling of infectious serum samples), the PCR products amplified using pfu polymerase (Promega) were first TA cloned into Zero Blunt TA vector (Invitrogen Inc., USA), followed by cloning into pCDNA3.1+ after digestion. Please refer to (Appendix Figure 9.2), for a graphical understanding of the cloning strategy. Essentially, PCR fragments 1 and 2 were both TA cloned. Sequencing was performed to ensure proper cloning and relative sequences compared to database. Using MluI, fragment 1 containing an internal XbaI RE site was cloned into pcDNA3.1+, prior to the MCS. Upon confirmation of directionality through sequencing, Fragment 2 was digested from TA vector and ligated at the XbaI site by normal cloning procedures. Sequencing was again performed to ensure the orientation of insertion.

Replication competency was checked by transient transfection of the cloned replicative genomes of the various genotypes and subjecting them to semi-quantitative IMX (Abbott Laboratories) analysis as well as quantitative Realtime RT-PCR of the precore region.

### 2.1.5 IMX Analysis

The efficiency of our cell-based viral replication system had to be established. In particular, we made use of the IMX system (Abbott Laboratories) utilizing their specific kit, HBsAg (V2) kit for the qualitative and semi-quantitative analysis of effective HBV viral replication.

Abbott Laboratories' IMX system makes use of a Microparticle Enzyme Immunoassay (MEIA) technology for detecting Hepatitis B surface antigen (HBsAg). This assay works on the specific interaction of the supplied mouse monoclonal anti-HBs coated microparticles for introduced HBsAg. Upon interaction, biotinylated anti-HBs were added to the antibody-antigen mixture to form "sandwich" of antibody-antigenantibody complex. This "sandwich" complexes were then bound to a glass fiber matrix and reacted with anti-biotin (Alkaline phosphatase conjugated). After unbound materials were removed by washing, 4-Methylumbeliferyl phosphate were added as substrate and measured by the IMX MEIA optical assembly. For our case, cells transfected with HBV replicative genomes were subjected to incubation at $37{ }^{\circ} \mathrm{C} 5 \% \mathrm{CO}_{2}$ for 48 hours for HBV replication and HBsAg secretion. Spent medium, containing secreted HBsAg were then collected and clarified of cell / cell debris by centrifugation at $14,000 \mathrm{rpm}$ for 15 min at 4 ${ }^{\circ} \mathrm{C} .160 \mu \mathrm{~L}$ of the supernatant were then applied to the carousel in conjunction with the HBsAg positive and negative controls (Abbott Laboratories). The process is semiautomated and readouts are printed for analysis. Effective weekly and monthly maintenance tasks dictated were carried out to ensure the accuracy of the readout. Also, due to the infrequency of use, the machine is calibrated before each run to ensure consistency in readouts. This analysis have been used in hospital clinical laboratories for identification of HBV chronic carriers by surface antigen detection of patient's serum.

### 2.1.6 Genotypes of $\mathbf{H B x}$

HBx gene of HBV genotypes B and C were amplified from the extracted viral DNA and cloned individually into pCDNA3.1+ (Invitrogen) via the EcoRV (underlined)
and NotI (bold) sites in the Multiple Cloning Site (MCS), making use of primers P7 [5'ATG ATA TCA TGG CTG CTA GGC TGT GCT GCC-3'] and P8 [5’-AAG CGG CCG CTT AGG CAG AGG TGA AAA AGT T-3']. After cloning, the plasmids were sequenced and compared to archived HBX sequences in NCBI. HBx gene of HBV genotypes B and C were also cloned into pEGFP-C1 (Clontech) within the MCS using HinDIII (underlined) designed in Primers P9 [5'-AAA AGC TTT TAT GGC TGC TAG GCT GTG CT $-3^{\prime}$ ] and Sall in P10 [5'-AAG TCG ACT TAG CCA CAG GTG AAA AGT -3']. Constructs of HBx of genotype A of both pCDNA3.1+ and pEGFP-C1 have been previously constructed in our laboratory.

### 2.1.7 Site-Directed Mutagenesis

Site-directed mutagenesis are important tools in molecular biology, allowing the study of mutations, natural or deliberate, on key essential proteins. This often leads to a clue of their downstream function after comparing differences between wild type and mutant protein. .

Site-Directed Mutagenesis reactions were performed using QuikChange SiteDirected Mutagenesis kit (Stratagene). Essentially, oligonucleotides were designed, with 17-18 nucleotides flanking the site of mutation. As per manufacturer's instruction, PCR were carried out using the provided reagents on a $1: 50$ diluted plasmid DNA of interest. The following reaction was set up: $5 \mu \mathrm{l}$ of 10 X Reaction buffer ( $100 \mathrm{mM} \mathrm{KCl}, 100 \mathrm{mM}$ $\left(\mathrm{NH}_{4}\right)_{2} \mathrm{SO}_{4}, 200 \mathrm{mM}$ Tris-HCl pH 8.8, $20 \mathrm{mM} \mathrm{MgSO} 4,1 \%$ Triton X-100 and $1 \mathrm{mg} / \mathrm{ml}$ nuclease-free bovine serum albumin) 50 ng of dsDNA template, 125 ng each of forward
and reverse oligonucleotides designed, $1 \mu \mathrm{l}$ of dNTP mix and topped up to $50 \mu \mathrm{l}$ using RNase-free water. $1 \mu \mathrm{l}$ of $p f u$ Turbo DNA polymerase ( $2.5 \mathrm{U} / \mu \mathrm{l}$ ) were last added and PCR was carried out. The cycling conditions are as described: $95{ }^{\circ} \mathrm{C}$ for 30 seconds followed by 18 rounds of cycling at $95{ }^{\circ} \mathrm{C}$ for $30 \mathrm{sec}, 55^{\circ} \mathrm{C}$ for $30 \mathrm{sec}, 68{ }^{\circ} \mathrm{C}(1 \mathrm{~min} / \mathrm{kb}$ of plasmid length).

Upon completion of PCR reaction, the parental strain was removed by the addition of $1 \mu \mathrm{l}$ of $\operatorname{DpnI}(\mathrm{NEB})$ and subjected to digestion for 2 hours at $37^{\circ} \mathrm{C} .2 \mu \mathrm{l}$ of the DpnI digested product, containing successful mutation were then transformed into DH5 $\alpha$ bacterial cells and positive mutation were detected by site-specific sequencing using oligonucleotides designed 100-200 bp upstream of the mutation site.

### 2.2 Cell Culture and Transfection

### 2.2.1 Cell Types

HepG2 (ATCC), Huh7, Chang Liver cells (ATCC-CCL13) and HEK293T (ATCC) were maintained and passaged in Gibco's Minimal Essential Medium Eagle (MEM) (Invitrogen), supplemented with $10 \%$ fetal bovine serum (FBS), $1 \%$ antimycotic (penicillin, streptomycin and amphotericin) at $37^{\circ} \mathrm{C}$ with controlled $\mathrm{CO}_{2}$ of $5 \%$. In particular, HepG2 is maintained for only 20-30 cycles after thawing and a new tube is thawed. This is to ensure the highest possible transfection efficiency as it has been shown that old cultures showed lower transfection efficiency (unpublished data). For all cell lines used, they were passaged at approximately $80-90 \%$ confluency to maintain a healthy state of growth for usage in our experiments.

### 2.2.2 Long Term Storage of Cells

Cells were grown to $80-90 \%$ confluency ( $\sim 2 \times 10^{7}$ cells) in 100 mm tissue culture dish. The spent culture medium was aspirated and cells were washed twice with 1 X PBS, pH 7.2 (Invitrogen Inc., USA). 2 X Trypsin / EDTA were then used for resuspension of the adherent cells. The reaction was stopped by addition of MEM (10 \% FBS) and cells were pelleted by centrifugation at $1,700 \mathrm{rpm}$ for 1 min at room temperature. The supernatant was aspirated and the pellet resuspended in 2 ml of storage medium, MEM (10 \% FBS, 10\% DMSO). The resuspended culture was then aliquoted into two 2 ml sterile plastic ampoule with screw cap. The ampoule was placed in styrofoam box and placed into $-80^{\circ} \mathrm{C}$ freezer overnight so as to ensure slower freezing rate. The frozen cells were rapidly transferred into liquid nitrogen tank for long term storage.

Cells can be thawed by swirling a frozen ampoule from the liquid nitrogen storage, in a $37^{\circ} \mathrm{C}$ water bath for approximately 1.5 min . The contents of the ampoule were then added to 9 ml of fresh MEM (10 \% FBS) and mixed by pipetting to dilute the DMSO content which might be detrimental to cell growth. The DMSO were removed by centrifugation at $1,700 \mathrm{rpm}$ for 1 min at room temperature and aspiration of the supernatant. Cell pellet was then resuspended in 12 ml of prewarmed MEM ( $10 \% \mathrm{FBS}, 1$ \% anti-mycotic) and applied to a 100 mm culture dish. Cells freshly thawed were passaged for a minimum of two cycles prior to any experimental usage to remove residual cryopreservative, DMSO.

### 2.2.3 Counting of Cells (for Experimental Seeding)



Figure 2.1: The above depicts cell counting device, Haemocytometer used. Top panel shows the application of the cover slide followed by addition of the sample. Bottom panel shows the magnified view observed under phase contrast microscope at 20 X magnification. Cells were counted at the 4 corners as well as the center portion and averaged.

## Source (http://www.ruf.rice.edu /~bioslabs/methods/microscopy/cellcounting.html)

Cell counting is made use of for all mammalian cell experiments to ensure consistency and a Haemocytometer (Figure 2.1) was used for this purpose. Precision coverslip was placed onto the top of a haemocytometer so as to cover the two ruled areas, each containing 9 large squares of the dimension of $0.1 \mathrm{~mm}^{3}$ each. It was pressed down gently until Newton's rings were visible. Trysinized cells were resuspended in appropriate MEM. This was then diluted 4 X using 1 X PBS and $50 \mu \mathrm{l}$ was then applied
at the groove allowing the sample to flow in under capillary action to cover the ruled areas. Cells in 5 boxes, 4 corner boxes and the center box, were counted. This was then averaged and multiplied by the dilution factor of 4 , followed by multiplication by $10^{4}$ to give the number of cells / ml.

### 2.2.4 Transfection of Cell Culture

Transfections were performed using Effectene Transfection Reagent (QIAGEN). 16-18 hours prior to transfection, $2.5 \times 10^{6}$ cells were seeded in $75 \mathrm{~cm}^{2}$ culture flasks or 100 mm tissue culture dishes. After incubation, the cells were transfected with $2 \mu \mathrm{~g}$ of various constructed plasmids processed as per manufacturer's instructions. Briefly, $2 \mu \mathrm{~g}$ of plasmids of interest, were reacted with $16 \mu \mathrm{l}$ of Enhancer (1 DNA : 8 Enhancer) by pulse-vortexing and incubated for 5 min . This was followed by addition of $50 \mu \mathrm{l}$ of Effectene Transfection Reagent (optimized for our cell line, unpublished data) and incubated for a further 10 min . Following that, 1 ml of pre-warmed culture medium (37 ${ }^{\circ} \mathrm{C}, 5 \% \mathrm{CO}_{2}$ ) were added to the transfection mix, pipetted twice to mix, and applied to the cells. Two hours post-transfection, the transfection medium were removed and rinsed with PBS twice, before the addition of fresh prewarmed culture medium. Transfected cells were maintained at $37{ }^{\circ} \mathrm{C}$ with $5 \% \mathrm{CO}_{2}$ for 48 hours followed by required experiments such as protein extraction.

### 2.2.5 Amaxa Nucleofector Transfection

HepG2 are one of the groups of hard to transfect mammalian cells. Various methods have been use but one of the methods that yielded the highest efficiency was the Amaxa nucleofector.

For high efficiency transfection of HepG2 cells (ATCC), the Nucleofector transfection technology (Amaxa Biosystems) was utilized. As per manufacturer's instruction, $1.5 \times 10^{6} \mathrm{HepG} 2$ cells were resuspended in $100 \mu \mathrm{~L}$ of Nucleofector Solution V. This was followed by the addition of $2 \mu \mathrm{~g}$ of intended plasmid DNA (Optimized by our lab). The mixture was then transferred carefully into the provided cuvettes for electroporation using Nucleofector (Note that cells should not be left for more then 15 min in the nucleofector mix). Programme T-28 was used for high efficiency transfection while Programme H-22 was used for high cell survival depending on the required experiment. Upon completion of transfection programme, the transfected cells were then mixed with $500 \mu \mathrm{~L}$ of pre-conditioned medium $\left(37{ }^{\circ} \mathrm{C}, 5 \% \mathrm{CO}_{2}\right)$ and transferred carefully onto 60 mm tissue culture dishes with supplied pipettes. Transfected cells were then allowed to incubate at $37{ }^{\circ} \mathrm{C} 5 \% \mathrm{CO}_{2}$ for $16-48$ hours, prior to various required experiments.

### 2.2.6 In situ Hybridization

Cells grown on coverslips or glass-bottom dishes (transfected or untransfected) were first washed 3 X in 1 X PBS (prewarmed to $37^{\circ} \mathrm{C}$ ) for 1 min . They were then fixed in Fixation buffer (3\% Paraformaldehyde / 1 X PBS) for 20 min. Fixation buffer were
aspirated and excess buffer removed by washing in 1 X PBS for 10 min . Cells were then permeabilized using Permeabilization buffer ( $0.2 \%$ TritonX-100 / 1 X PBS) for 10 min . Blocking was performed using 10 \% FBS / 0.1 \% TritonX-100 / 1 X PBS for 10 min . Blocking buffer were aspirated and cells were washed twice with 1 X PBS for 1 min followed by addition of primary antibodies (reconstituted $1: 1000$ to $1: 5,000$, in $1 \%$ TritonX-100 / 1 X PBS). After incubation for 2 hours at $37{ }^{\circ} \mathrm{C}$ or overnight at $4{ }^{\circ} \mathrm{C}$, the cells were washed 2 X with 0.1 \% TritonX-100 / 1 X PBS for 15 min . Secondary antibodies with suitable fluorophore (Alexa fluor 488 nm or 546 nm ) (Molecular Probes) were added (diluted 1: 1000 using $1 \%$ TritonX-100 / 1 X PBS) and incubated in the dark at R.T. for 1 hour. After incubation, cells were washed 2 X with $0.1 \%$ TritonX-100 / 1 X PBS for 15 min . The washing buffer was removed by aspiration and the stained cells were dried overnight at R.T. For coverslips, mounting media (Invitrogen Inc., USA) were used which consists of DAPI staining (for nuclear staining). The prepared slides were analyzed under fluorescent microscope, Olympus IX71 and/or Carl Zeiss confocal microscope. In the case of single staining (e.g. actin), Phalloidin tagged with Alexa fluor 488 nm or 546 nm were used and only a single wash were required followed by mounting.

### 2.3 Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

### 2.3.1 SDS-PAGE

SDS-PAGE was routinely made use of, to separate proteins by molecular weight regardless of their intrinsic electrical charges (Shapiro et. al., 1967). Specifically, the Laemmli (1970) discontinuous system is routinely made use of. Briefly, proteins are
stacked in a stacking gel before entering into a separating gel to ensure good resolution of the gel.

### 2.3.2 Preparation of SDS-PAGE

$12 \%$ separating gel were made use of and prepared using Bio-Rad mini-protean electrophoresis system. For two 1 mm thick gels, 10 ml of separating gel solution and 5 ml of stacking gel solution were prepared. The separating gel, consisting of 4 ml of monomer solution ( $29 \%$ acrylamide : $1 \%$ bisacrylamide) (Bio-Rad), 2.5 ml of 4 X separating gel buffer ( 1.5 M Tris- $\mathrm{HCl}, \mathrm{pH} 8.8$ ), 0.1 ml of $10 \%$ SDS, 0.1 ml of $10 \%$ ammonium persulfate (APS), 3.3 ml of distilled water and $5 \mu \mathrm{l}$ of $\mathrm{N}, \mathrm{N}, \mathrm{N}^{\prime}, \mathrm{N}^{\prime}$ -tetramethyl-ethylenediamine (TEMED), was laid first. To ensure a straight surface, 0.2 ml of water-saturated butanol (Preparation at the end of section) was added. After setting of the separating gel for a minimum of 15 min at room temperature, the water-saturated butanol was removed by washing using distilled water. The excess water was drained properly prior to casting of the stacking gel. A $5 \%$ stacking gel solution containing 0.67 ml of monomer solution ( $29 \%$ acrylamide : $1 \%$ bisacrylamide), 0.83 ml of 4 X stacking gel buffer ( 0.5 M Tris- $\mathrm{HCl}, \mathrm{pH} 6.8$ ), $50 \mu \mathrm{l}$ of $10 \%$ SDS, $50 \mu \mathrm{l}$ of $10 \%$ APS, 3.4 ml of distilled water and $5 \mu \mathrm{l}$ of TEMED was prepared, mixed properly and applied above the separating gel. Combs of appropriate thickness were used and the stacking gel was allowed to set for a minimum of 15 min at room temperature. Prior to run, the comb was removed and the formed wells were washed substantially with distilled water to remove excess unpolymerized monomer solution.

The gel run was setup by placing the casted gels with short plates facing inwards towards the inner buffer chamber. The inner buffer chamber was slowly filled with fresh 1 X SDS running buffer (0.025 M Tris, 0.192 M glycine, pH 8.3, $0.1 \%$ SDS). Various protein samples of interest were quantified, mixed with 6 X SDS sample buffer and boiled for 10 min . Molecular weight rainbow markers RPN 755 and RPN 756, were purchased from GE Healthcare and used on low molecular weight detection (<45 kDa) and high molecular detection (up to 200 kDa ) respectively. The samples were then pulsed down and applied to each well. The initial stacking runs were performed at 80 V until the samples have entered the separating gel. For the separation phase, the gels were subjected to 150 V and power was turned off once the dye had visually reached the bottom of the setup. The gels were then either stained with Coomassie Blue Staining (50 \% (v/v) methanol, 0.05 \% (w/v) Brilliant Blue R-250, 10 \% (v/v) glacial acetic acid, 40 \% distilled water) overnight prior to destaining using destaining solution (5 \% (v/v) methanol, $7 \%(\mathrm{v} / \mathrm{v})$ glacial acetic acid, $88 \%$ distilled water) for approximately 4 hours. The SDS-PAGE could also be subjected to Western analysis by first soaking for 15 min in transfer buffer ( 25 mM Tris, 192 mM glycine, $10 \%(\mathrm{v} / \mathrm{v})$ methanol) followed by semidry transfer onto prewetted nitrocellulose and activated polyvinylidene difluoride (PVDF) membrane.

Preparation of water-saturated butanol: 40 ml of 2-Butanol (Fisher) were added to 10 ml of distilled water. They were then thoroughly mixed and allowed to settle overnight. The top layer consisting of the water-saturated butanol was used for leveling of separating gels and also to remove any surface bubbles which might affect the run.

### 2.3.3 Western Transfer (Semi-dry system)

SDS-PAGE gel was transferred via Bio-Rad's Semidry transfer system onto 0.45 $\mu \mathrm{m}$ nitrocellulose membrane (GE Hybond) or $0.2 \mu \mathrm{~m}$ PVDF membrane (Bio-Rad). Nitrocellulose membranes were pre-soaked with transfer buffer ( 25 mM Tris, 192 mM glycine, $10 \%(\mathrm{v} / \mathrm{v})$ methanol). On the other hand, PVDF membrane were first activated by soaking in absolute methanol ( 15 sec ) followed by two washes in distilled water ( 5 min each) followed by soaking in transfer buffer ( 25 mM Tris, 192 mM glycine, $10 \%$ (v/v) methanol) for 15 min prior to transfer. Usually, the settings for the transfer is fixed at constant voltage of 21 V with varying timings from 15 min to 50 min depending on the molecular weight of the protein of interest (Refer to Figure 2.2 for gel transfer setup). After completion of the transfer, nitrocellulose membrane was subjected to Ponceau S staining ( $0.1 \%(\mathrm{w} / \mathrm{v})$ Ponceau S, $5 \%(\mathrm{v} / \mathrm{v})$ glacial acetic acid, $95 \%$ distilled water) to determine transfer efficiency. Ponceau S stain of the transferred proteins were then washed off by distilled water.


Figure 2.2: Schematics of the setup of Bio-RAD semidry system.

### 2.3.4 Western Analysis

Transferred membranes were then subjected to blocking in $5 \%$ non-fat milk/ 1 X PBS for 1 hr R.T. or $4{ }^{\circ} \mathrm{C}$ overnight with rotation. After blocking, the specific primary
antibody, in $3 \%$ non-fat milk / 1 X PBS, was reacted for 1 hour R.T. Non-specific interactions were then removed by 3 washes of 10 min utilizing cold 1X PBS/ $0.005 \%$ Tween-20. Secondary antibody tagged with Horseradish Peroxidase (HRP), in $3 \%$ nonfat milk / 1 X PBS, was then reacted for another hour if required. This was followed subsequently by another 3 washes of 10 min with cold 1 X PBS / $0.005 \%$ Tween-20. The membrane is then ready for ECL using SuperSignal West Pico chemiluminescent substrate (Pierce). Essentially, an equal volume of stable peroxide solution and luminol / enhancer solution were mixed and applied to the membrane processed above. The substrate was incubated for 1 min R.T., after which excess substrates were decanted and the membrane wrapped in Cling wrap.

Detection of ECL was performed either using Pierce CLxposure X-ray film (blue color) or GE healthcare's ECL hyperfilm (transparent). The signals on the membrane were exposed to the X-ray film for varying durations, dependant on the antibodies and intrinsic concentration of the protein of interest. Typically, an exposure lasts between 15 sec to 2 hours. Developing and fixing reagents were purchased from Kodak and diluted 5 X each in distilled water prior to use. Film was developed manually and varying timings were adjusted to obtain properly exposed results clear of background. Essentially, after exposure, the films were placed in the diluted developer solution and agitated until visualization of the targeted band. The films were then removed and washed with distilled water to remove excess developer. The films were then fixed by agitation in the diluted fixing solution for approximately 2 min .

Preparation of $1 \mathbf{X}$ PBS: $\operatorname{PBS}\left(137 \mathrm{mM} \mathrm{NaCl}, 2.7 \mathrm{mM} \mathrm{KCl}, 4.3 \mathrm{mM} \mathrm{Na} 2 \mathrm{HPO}_{4} .7 \mathrm{H}_{2} \mathrm{O}\right.$, $1.4 \mathrm{mM} \mathrm{KH} 2_{2} \mathrm{PO}_{4}$. Briefly, 8 g of $\mathrm{NaCl}, 0.2 \mathrm{~g}$ of $\mathrm{KCl}, 1.15 \mathrm{~g}$ of $\mathrm{Na}_{2} \mathrm{HPO}_{4} .7 \mathrm{H}_{2} \mathrm{O}, 0.2 \mathrm{~g}$ of $\mathrm{KH}_{2} \mathrm{PO}_{4}$ ) was added and dissolved in 800 ml of sterile distilled water. pH was adjusted to 7.2 and distilled water was added to 1 L for working 1 X PBS.

### 2.3.5 Reprobing of PVDF Membrane

For stripping of probed membrane for repeated Western blot analyses, we made use of Restore Western Stripping solution (Pierce). Essentially, probed membrane were first soaked in distilled water at $4{ }^{\circ} \mathrm{C}$ prior to stripping. After decanting of the distilled water, enough stripping solution was added to cover the entire membrane and incubated at R.T. for $15-30$ min depending on the intensity of the previous Western. Following the stripping, the membrane was subjected to washing with $1 \mathrm{X} \mathrm{PBS} / 0.05 \%$ Tween-20 for 10 min . Prior to probing by another antibody, Substrate was added as per Western and the membrane was subjected to ECL to eliminate any residual luminescence from the previous probe. Once cleared, the membrane was washed in 1 X PBS with $0.05 \%$ Tween-20 prior to further Western analysis.

### 2.3.6 Phosphorylation Protein Lysates Preparation

HEK293T cells were transfected with the various constructs using Effectene Transfection Regent and incubated in serum-free medium for 18 hours. The cells were then supplemented with $5 \%$ FBS 4 hours prior to protein collection. Cells were first washed with ice-cold 1 X PBS prior to addition of lysis buffer ( 50 mM Tris-Cl pH8.0, $150 \mathrm{mM} \mathrm{NaCl}, 1 \mathrm{mM}$ EDTA, $1 \% \mathrm{NP} 40,50 \mathrm{mM}$ NAF, $1 \mathrm{mM} \mathrm{Na}_{3} \mathrm{VO}_{4}$ ) supplemented with

EDTA-free Complete ${ }^{\text {TM }}$ protease inhibitor cocktail (Roche). Lysates were collected and subjected to brief sonication followed by clarification at $15,300 \mathrm{rpm}$ for 15 min at $4^{\circ} \mathrm{C}$. The supernatant containing the protein of interest were quantified by use of Bradford protein assay (Bio-Rad) and $25 \mu \mathrm{~g}$ were used for Western analysis. Anti-ERK1/2 and anti-phosphorylated ERK1/2 antibodies were obtained from BD transduction laboratories while anti-AKT and anti-phosphorylated-AKT were obtained from Cell Signaling Technology.

### 2.3.7 Gel Overlay Assay

Proteins were ran on a $12 \%$ SDS-PAGE and transferred on a nitrocellulose membrane as per earlier sections. Potential interacting proteins (either bacterial expressed or mammalian expressed proteins) with detectable molecular tags (e.g. HIS, HA or FLAG) were diluted in 1 X PBS and applied for interaction at $4{ }^{\circ} \mathrm{C}$ for 4 hr with rotation. Following this, non-specific interactions were removed by 2 washes in $1 \mathrm{XPBS} / 0.05 \%$ Tween-20. The following steps are as per Western protocol using antibodies specific for the tagged interacting protein.

### 2.4 2-Dimensional Electrophoresis (2DE)

2-Dimensional Electrophoresis (2DE) has become an important tool for biologists in the recent years. This technique has been harnessed especially to understand the difference in protein expression profiles between healthy and carcinoma cells (Seow et. al., 2000, Liang et. al., 2002) as well as to understand any adverse effects on potential drug treatments (Cheng et. al., 2005). Essentially, proteins were first separated by virtue
of their isoelectric points $(p I)$ through the use of immobilized pH gradient strips. The separated proteins were then subjected to the second dimension through a separating gel. This allows the proteins of different molecular weight from similar pI range to be separated. This sequential separation allows for the increased separation between proteins allowing for ease of identification by mass spectrometry.

### 2.4.1 Total Proteins Extraction for 2D Electrophoresis

48 hours post-transfection, cultures were trypsinized using 5 ml of 2 X Trypsin/ EDTA. After centrifugation, cells were then spun down at 2,000 rpm. Excess medium were removed and cells were then washed with 1 XPBS , pH 7.2 three times and pelleted by centrifugation at $2,000 \mathrm{rpm}$ for 5 min at $4^{\circ} \mathrm{C}$. This was followed by cell lysis in the presence of 200-600 $\mu \mathrm{l}$ of Lysis buffer containing, 7 M urea, 2 M Thiourea, $1 \%$ nuclease mix (GE Healthcare) and $4 \%$ EDTA-free Complete Proteinase Inhibitor (Roche). Briefly, the cells in lysis buffer were vortexed for 1 min vigorously and subjected to clarification by centrifugation at $15,300 \mathrm{rpm}, 4{ }^{\circ} \mathrm{C}$ for 1 hour. Supernatant, containing total proteins, were aliquoted and stored at $-80{ }^{\circ} \mathrm{C}$ prior to protein quantification and Isoelectric Focusing (IEF).

### 2.4.2 1st Dimension Electrophoresis

Total proteins were separated by their isoelectric points $(p I)$ at high voltage. Extracted proteins were first quantified by 2D Quant Kit (GE healthcare) modified from Lowry's method. Essentially, the kit allows for accurate measurement of proteins in buffers with high molarity of various components, compared to traditional protein
quantification kits. This is achieved by a precipitation of the proteins from the interfering components (e.g. high molarity of urea and thiourea) of the lysis buffer. The precipitated proteins were then re-dissolved and quantified. $80 \mu \mathrm{~g}$ of total proteins were used for analytical gels while $200 \mu$ g were used for preparative gels. 18 cm IEF strips pH 3-10 NL (GE Healthcare) were rehydrated overnight using $350 \mu \mathrm{l}$ of rehydration buffer containing 7 M Urea, 2 M Thiourea, 10 mM DTT and 10 \% IPG buffer pH 3-10NL (GE Healthcare). Samples were then cup loaded onto the rehydrated strips and IEF was performed according to the following steps: (1) $200 \mathrm{~V}, 100 \mathrm{Vh}$; (2) 500 V , 250 Vh ; (3) $1000 \mathrm{~V}, 500$ Vh; (4) 1000-8000 V gradient, 2250 Vh ; and (5) $8000 \mathrm{~V}, 32000 \mathrm{Vh}$ (Liang et. al.., 2002). Focused Gel strips were immediately stored at $-80^{\circ} \mathrm{C}$ or immediately subjected to 2 nd dimension electrophoresis using SDS-PAGE.

### 2.4.3 2nd Dimension Electrophoresis

Focused Gel strips were first equilibrated with 10 ml Equilibration Buffer ( 6 M Urea, 50 mM Tris-HCl pH 8.8, $30 \%(\mathrm{v} / \mathrm{v})$ glycerol, $2 \%$ SDS and bromophenol blue) in 2 step; (1) Reduction of disulphide bonds by the addition of DTT (1 \% w/v) for 15 minutes followed by (2) Alkylation by addition of Iodoacetamide ( $2.5 \% \mathrm{w} / \mathrm{v}$ ) for the subsequent 15 minutes. SDS-PAGE was then carried out using $10 \% 1.5 \mathrm{~mm}$ thick polyacrylamide gels with Bio-Rad's Protean II XL Cell or GE healthcare, Ettan DaltSix. Electrophoresis was then carried out with a constant 30 mA current per gel. Following gel run, each gel was fixed using $40 \%(\mathrm{v} / \mathrm{v})$ ethanol / $20 \%$ (v/v) glacial acetic acid, overnight at 30 rpm before subjecting them to Silver Staining (As per PlusOne Silver Staining Kit, GE healthcare)

### 2.4.4 Data Analysis

Silver-stained gels were documented using ImageScanner II (GE Healthcare) and the comparison between the gels, were made both visually as well as through the use of ImageMaster ${ }^{\text {TM }}$ 2D Platinum software (GE Healthcare). Interesting spots were picked up and trypsinized followed by Mass Spectrometry utilizing Matrix-Assisted Desorption / Ionization Time-of-Flight (MALDI-TOF) performed by Protein and Proteomics Center (PPC), National University of Singapore. Peptide fingerprints obtained were then sent to database such as Mascot (http://www.matrixscience.com/) and Protein Prospector (http://prospector.ucsf.edu/) for identification of protein of interest. Comparisons were also made with 2D database of liver cell lines at Swiss2D-PAGE.

### 2.5 Bacterial Protein Expression and Purification

### 2.5.1 Bacterial Expression

Constructs of bacterial expression vectors, pGEX or pET vector(s) were transformed into BL21 (DE3) bacterial strain. Isolated colonies were selected and grown in 5 ml of LB with selective antibiotics overnight at $37{ }^{\circ} \mathrm{C} 250 \mathrm{rpm}$. The culture were then scaled up 100 X accordingly depending on the final volume required ( 1 ml culture into 100 ml fresh Luria Bertani (LB) broth, with selective antibiotics of $100 \mu \mathrm{~g} / \mathrm{ml}$ ampicillin or $30 \mu \mathrm{~g} / \mathrm{ml}$ kanamycin). The seeded cultures were then subjected to further growth at $37{ }^{\circ} \mathrm{C} 250 \mathrm{rpm}$ until cell density of $\mathrm{OD}^{600 \mathrm{~nm}} 0.6$ or 1.0 were achieved. 100 mM filter-sterilized ( $0.2 \mu \mathrm{~m}$ filter) Isopropyl- $\beta$-D-thiogalactopyranoside (IPTG) (Fermentas) were then added in the range of 0.1 mM to 1 mM for induction of protein expression. The
cultures were then incubated in varying temperatures of 16,30 and $37{ }^{\circ} \mathrm{C}$ for varying hours ( 6,8 and 4 respectively) for protein expression. Protein lysates were then prepared either by Bacterial Protein Extraction Reagent (B-PER) (Pierce) for small scale extraction or by sonication. Basically, large cultures of bacteria ( $>100 \mathrm{ml}$ ) were spun down at 5,500 rpm for 15 min at $4^{\circ} \mathrm{C}$, and pellets were resuspended with appropriate amount of lysis buffer ( 50 mM Tris-Cl $\mathrm{pH} 8.0,150 \mathrm{mM} \mathrm{NaCl}, 1 \%$ TritonX-100) supplemented with EDTA-free Complete protease inhibitor (Roche). Cells were then subjected to sonication and clarified at $15,300 \mathrm{rpm}$ for 60 min at $4{ }^{\circ} \mathrm{C}$. The supernatant, containing the soluble protein were then retained for quantification (Bio-Rad's Quick Start Bradford Protein Assay) and use in subsequent experiments. Insoluble pellet was resuspended in 2 X SDS sample buffer ( 125 mM Tris-Cl pH 6.8, $4 \%$ SDS, $10 \%$ 2-mercaptoethanol / 150 mM DTT, $20 \%$ glycerol, $0.01 \%$ Bromophenol blue) for use in case the protein of interest turned out to be insoluble. Protein lysates were stored at $-80^{\circ} \mathrm{C}$ prior to use.

Preparation of LB broth and agar: 5 g of Bacto Tryptone (BD Bioscience), 5 g of NaCl (Sigma Aldrich) and 2.5 g of Bacto Yeast Extract (BD Bioscience) were added and made up to 500 ml with deionized water. For LB agar, an additional 7.5 g of Bacto Agar (BD Bioscience) was added to the above mixture. The mixtures were then autoclaved and cooled to $\sim 45-50{ }^{\circ} \mathrm{C}$ prior to addition of required antibiotics. LB agar were then poured onto petri dish, and stored in $4{ }^{\circ} \mathrm{C}$ for less then 2 months.

### 2.5.2 GST-fusion Protein Purification

GST-fusion proteins were subjected to purification using Microspin GSTpurification modules (GE Healthcare). Essentially, quantified GST-fusion proteins were mixed with the sepharose 4 B beads for 30 min with head to end rotation at $4^{\circ} \mathrm{C}$. The noninteracting proteins (non-GST fusion) were removed by centrifugation at $3,000 \mathrm{rpm}$ for 1 min R.T. Non-specific interactions were then removed with 3 washes of ice-cold 1 X PBS ( $600 \mu \mathrm{l})$. Reduced glutathione were then added and rotated for another $15 \min 4{ }^{\circ} \mathrm{C}$ prior to elution of purified sepharose 4 B beads. Eventually, the GST-fusion proteins captured by the sepharose 4B beads were then collected by centrifugation at $3,000 \mathrm{rpm} 1$ min R.T. For higher purity, the purified GST-fusion protein could be subjected to a second round of purification using the above mentioned procedure.

Larger scale GST-fusion proteins (> $600 \mu \mathrm{l}$ or $300 \mu \mathrm{~g}$ ) were subjected to purification using self-packed 15 ml falcon tube with appropriate amount of sepharose 4B beads (GE Healthcare). Similar steps to the microspin columns mentioned in the earlier paragraph were used except scaling up of wash buffer (1 X PBS) volumes.

### 2.5.3 GST-pulldown Interaction Assay

As above, the GST-fusion protein of interest were interacted with Glutathione Sepharose 4B beads by rotation at $4{ }^{\circ} \mathrm{C}$ for 30 min . Excess unbound proteins were removed by 3 X washes with 1 X PBS. The cell lysates or expressed protein for interaction with the bound proteins were then introduced and allowed to interact by subjecting to rotation for 4 hours. Non-interacting proteins were then removed by
centrifugation at 3000 rpm 1 min . This was then subjected to 3 subsequent washes in 1 X PBS / 0.005 \% Tween-20. Finally, elution was performed using reduced glutathione (10 mM glutathione in 50 mM Tris- Cl pH 8.0).

### 2.6 Panomics TranSignal SH3 Domain Arrays

TranSignal SH3 domain arrays were purchased from Panomics. Essentially, SH3 domains from various known proteins were expressed and spotted in duplicates on a nitrocellulose membrane. GST Fusion protein with six Histidine-tag were generated for this purpose. GST-PXXP-his were generated from Genotypes A, B and C and used for interaction analysis with the Panomics membrane. $15 \mu \mathrm{~g}$ of Glutathione Sepharose purified GST-fusion proteins were interacted with the membrane as per manufacturer's instructions. Non-interacting proteins were washed off with the provided wash buffer and interacting proteins were detected by use of anti-Histidine antibodies provided with the membrane. Positive interactors of SH3 domain were shown by developing with ECL. Controls of the membrane was provided in the form of his peptides spotted in duplicates on the right and bottom of the membrane by the manufacturer.

### 2.7 Real-time Reverse Transcription-Polymerase Chain Reaction

### 2.7.1 Total RNA Extraction

Total RNA was extracted from transfected or untransfected mammalian cells using RNeasy mini kit (QIAGEN) using spin columns protocol as per manufacturer's instructions. Briefly, cells were pelleted by trypsinization and resuspended in $350 \mu \mathrm{l}$ or $600 \mu \mathrm{l}$ (> $5 \times 10^{6}$ cells) of Buffer RLT (proprietary to QIAGEN) ( 1 ml of Buffer RLT
supplemented with $10 \mu \mathrm{l}$ of $\beta$-mercaptoethanol). They were then subjected to homogenization for 30 sec followed by addition of an equal volume of $70 \%$ analytical grade ethanol. The mixture were then applied to the spin columns supplied (reloading if volume was bigger then $700 \mu \mathrm{l}$ ) and centrifuged at $13,200 \mathrm{rpm}$ for 30 sec . The RNA attached to the columns were then washed with $700 \mu \mathrm{l}$ of Buffer RW1 (proprietary to QIAGEN) and centrifugation at $13,200 \mathrm{rpm}$ for 30 sec . Two further washing with $500 \mu \mathrm{l}$ Buffer RPE (proprietary to QIAGEN) each was performed by centrifugation at 13,200 rpm for 30 sec and 2 min respectively. This was followed by a further centrifugation at $13,200 \mathrm{rpm}$ for 1 min to remove any residual Buffer RPE. Eventually, the total RNA was eluted using $30 \mu \mathrm{l}$ of RNase-free water (supplied) or DEPC-treated water. Total RNA were stored at $-70^{\circ} \mathrm{C}$ for long periods with minimal degradation.

### 2.7.2 RNA Quantitation

Total RNA were then quantified by measuring the absorbance using the UV spectrophotometer at $\mathrm{OD}^{260 \mathrm{~nm}}$ and $\mathrm{OD}^{280 \mathrm{~nm}}$. Yield was calculated by multiplication of $\mathrm{OD}^{260 \mathrm{~mm}}$ by $40 \mu \mathrm{~g} / \mathrm{ml}$ and a further multiplication by the dilution factor. Purity of the extracted RNA were calculated by division of $\mathrm{OD}^{260 \mathrm{~nm}}$ over $\mathrm{OD}^{280 \mathrm{~nm}}$ and a good value between 1.9 to 2.1 equates to good RNA quality.

### 2.7.3 QIAGEN OneStep Reverse Transcription - Polymerase Chain Reaction

Specific oligonucleotides were synthesized for particular genes of interest and reverse transcribed using QIAGEN One-Step RT-PCR kit. Briefly, total RNA were added (between 1 pg to $2 \mu \mathrm{~g}$ ) to a master mix containing $10 \mu \mathrm{l}$ of 5 X QIAGEN OneStep RT-

PCR buffer, $2 \mu \mathrm{l}$ of dNTP mix (containing 10 mM of each dNTP), $1.5 \mu \mathrm{l}$ of $20 \mathrm{mM}(0.6$ $\mu \mathrm{M})$ forward and reverse oligonucleotides respectively, $2 \mu \mathrm{l}$ of QIAGEN OneStep RTPCR Enzyme mix and reconstituted to a total volume of $50 \mu \mathrm{l}$ using RNase-free water. RT-PCR mixture were kept on ice and placed onto the thermal cycler only upon reaching $50{ }^{\circ} \mathrm{C}$.

RNA were subjected to an initial reverse transcription at $50{ }^{\circ} \mathrm{C}$ for 30 min , followed by $95{ }^{\circ} \mathrm{C}$ for 15 min for activation of the HotStarTaq DNA polymerase and inactivation of the reverse transcriptases. A 3-step cycling consisting of denaturation at $94{ }^{\circ} \mathrm{C}(1 \mathrm{~min})$, annealing at $50^{\circ} \mathrm{C}(1 \mathrm{~min})$ and extension at $72{ }^{\circ} \mathrm{C}$ from 1 min (increase accordingly - $1 \mathrm{~min} / \mathrm{Kb} \mathrm{DNA}$ ). This was allowed to cycle for 30 cycles followed by a final extension at $72{ }^{\circ} \mathrm{C}$ for 10 min . PCR products were then ran on a $1 \%$ agarose gel and visualized using ethidium bromide.

### 2.7.4 Real-time RT-PCR

Real-time RT-PCR was made use of to accurately quantify starting amount of target mRNAs instead of traditional RT-PCR. Real-time RT-PCR monitors the amplification of PCR product during cycling rather then the end product like traditional RT-PCR. SYBR Green, incorporation allows for detection of the double stranded DNA by intercalation. On excitation, fluorescence is emitted and detected. Quantification is performed by calculation of the fluorescence density during amplification cycles.
iSript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) were made use of for real-time quantification of starting gene template. Briefly, a pair of gene-specific oligonucleotides encompassing a 120 - 130 bp fragment was designed from the gene of interest (e.g. pre-Core region for replication intermediates determination). PCR reactions were optimized by the use of the QIAGEN OneStep RT-PCR kit to obtain minimal nonspecific banding and primer-dimer. Conditions were then used in the cycling of the Realtime RT-PCR reactions.

Briefly, the reactions were set up by addition of $25 \mu \mathrm{l}$ of 2 X SYBR green RTPCR reaction mix (containing 0.4 mM of each dNTP, magnesium chloride and i Taq DNA), $1.5 \mu \mathrm{l}$ each of forward and reverse primer $(10 \mu \mathrm{M}) .1 \mu \mathrm{l}$ of iScript Reverse Transcriptase (50 X iScript MMLV reverse transcriptase) for One-Step RT-PCR was added followed by addition of 10 ng of RNA, followed by addition of nuclease-free water up to $50 \mu \mathrm{l}$ total volume. These reaction mix were transferred to 0.2 ml white strip tubes (Bio-Rad) and covered with optical flat strip caps (Bio-Rad) and maintained in ice prior to run.

The Realtime PCR was carried out on a IQ5 multicolor Real-time PCR detection system (Bio-Rad) with the following cycling protocols: cDNA synthesis at $50^{\circ} \mathrm{C}$ for 10 min, iScript Reverse Transcriptase inactivation at $95{ }^{\circ} \mathrm{C}$ for 5 min , PCR cycling and detection for 40 cycles using 2-step protocol of $95^{\circ} \mathrm{C}$ for 10 sec and $55^{\circ} \mathrm{C}$ for 30 sec . The dissociation analysis was also carried out by the acquisition of fluorescent reading for one degree increase from $55{ }^{\circ} \mathrm{C}$ to $95{ }^{\circ} \mathrm{C}$. Experimental report, melt curve analysis and
threshold cycle number were extricated from the DNA IQ5 optical system software version 2.0 (Bio-Rad).

The fold changes were calculated by the below formula.
Sample $\Delta \mathrm{Ct}=\mathrm{Ct}_{\text {sample }}-\mathrm{Ct}_{\text {pactin }}$

$$
\Delta \Delta \mathrm{Ct}=\text { Sample } \Delta \mathrm{Ct}-\operatorname{Control} \Delta \mathrm{Ct}
$$

The fold of sample against control was calculated by $2^{\Delta \Delta C t}$.

### 2.8 Confocal Reflection Interference Contrast Microscopy (C-RICM)

### 2.8.1 Collagen Coating

In brief, $400 \mu \mathrm{l}$ stock solution of rat-tail collagen (BD Biosciences Inc., USA) at a concentration of $1 \mathrm{mg} / \mathrm{ml}$ in 0.012 N HCl was neutralized by the addition of $50 \mu \mathrm{l}$ of 0.1 M NaOH and $50 \mu \mathrm{l}$ of 10 X PBS (final collagen concentration of $0.8 \mathrm{mg} / \mathrm{ml}$ ). Glass coverslip (Fisher Inc., USA) was cleaned with a mixture of $30 \% 1 \mathrm{~N} \mathrm{NaOH}$ and $70 \%$ methanol in an ultrasonic bath for 20 minutes then washed in pure methanol for 15 minutes, autoclaved and sterilized under UV light for 30 minutes. The neutralized collagen solution was spread out evenly on the surface of glass coverslip with the use of a cell scraper. After 24 hours of collagen incubation at $4^{\circ} \mathrm{C}$, the coverslips were then washed with 1X PBS for three times and dried in air before use.

### 2.8.2 C-RICM

The system is based on a laser scanning confocal microscope (Pascal 5, Carl Zeiss, Germany) and is integrated with a temperature/ $\mathrm{CO}_{2}$ control chamber (Carl Zeiss, Germany). The illumination source is an Argon-ion laser with a maximum power of

1 mW and excitation wavelength of $488 \mathrm{~nm} .63 \times$ oil immersion objective (Neofluar, NA: 1.25) was used in this study. Immediately following the seeding of HepG2 cells infected with HBV or HepG2 cells with empty vector or normal HepG2 cells on collagen coated coverslip, a series of C-RICM images was taken to investigate the kinetics of adhesion contact area for adherent cells from 0 to 2.5 hours. Strong contact zone of the adherent cell appears as dark region on the image ZSM5 software (Carl Zeiss, Germany) and was used for image analysis. The contact area was determined with the drawing tool of the software which indicates the area of contact between the cell and the substrate. The error bar on cell deformation is originated from the standard derivation of at least three sets of experimental data (with at least 60 cells) under each condition.

### 2.8.3 Data Analysis

Briefly, the equilibrium geometry of a water-filled cell adhering on rigid substrate is modeled as a truncated sphere with a mid-plane radius $R$. Degree of deformation, $\sin \theta$ $=(a / R)=\alpha$ is an experimentally measurable parameter of cell geometry where $a$ is the contact zone radius and $\theta$ is the contact angle. $R$ and $a$ is measured by C-RICM and phase contrast microscopy, respectively. The cell wall is under a uniform equi-biaxial stress, $\sigma=T \varepsilon . T$ is the stress equivalent and is equal to $E h /(1-v)$ in a linear system under small strain where $E, h$ and $v$ is the elastic modulus, membrane thickness and Poisson's ratio, respectively. The average biaxial strain, $\varepsilon$, is directly calculated from experimental data including $R$ and $\alpha$ as follows:

$$
\begin{equation*}
\varepsilon=\frac{1}{2}\left[\frac{2+2\left(1-\alpha^{2}\right)^{1 / 2}}{4 / R^{2}-\alpha^{2}}-1\right] \tag{1}
\end{equation*}
$$

In the absence of external force, it was shown earlier that the adhesion energy, $W$, is

$$
\begin{equation*}
W=(1-\cos \theta) C \varepsilon+C \varepsilon^{2} \tag{2}
\end{equation*}
$$

Based on the experimental measurements of the mid-plane diameter $R$ (phase contrast microscope) and the radius of contact zone, $a$ (C-RICM), $W$ can be found by Eqs (1) and (2). Elastic modulus $E$ of HepG2 cell is taken as $2000 \mathrm{~N} / \mathrm{m}^{2}$ according to the experimental results obtained from AFM indentation.

### 2.9 Cloning

### 2.9.1 Chemically Competent Bacterial Cells Preparation.

A loopful of DH5a Escherichia coli was streaked onto a fresh LB agar plate (without selective antibiotics) and incubated at $37{ }^{\circ} \mathrm{C}$ overnight. Following that, a single isolated colony of the DH5 $\alpha$ E. coli was inoculated into 5 ml of LB broth (without selective antibiotics) and incubated with shaking at 250 rpm overnight in a $37^{\circ} \mathrm{C}$ shaking incubator overnight. After that, the bacteria culture was scaled up 100 times into 100 ml of fresh LB and grown to $\mathrm{OD}^{600 \mathrm{~nm}}$ of 0.3 to 0.4 . The culture in the exponential phase was then aliquoted into $2 \times 50 \mathrm{ml}$ pre-chilled centrifuge tubes and incubated on ice for 10 min . The bacteria cells were then pelleted by centrifugation for 5 min at $5,000 \mathrm{rpm}$ at $4{ }^{\circ} \mathrm{C}$. The spent media was then decanted and the bacterial cells pellet was resuspended in 30 ml of 0.1 M ice-cold $\mathrm{MgCl}_{2}$ thoroughly. The mixture was then centrifuged for 5 min at $5,000 \mathrm{rpm}$ in $4^{\circ} \mathrm{C}$. The supernatant was then decanted and the pellet was resuspended in 20 ml of 0.1 M ice-cold $\mathrm{CaCl}_{2}$ and incubated on ice for 30 min . This was followed by a centrifugation for 5 min at $5,000 \mathrm{rpm}$ in $4^{\circ} \mathrm{C}$. Again, supernatant was decanted and each
pellet was finally resuspended in 1.5 ml of ice-cold $0.1 \mathrm{M} \mathrm{CaCl}_{2} / 15 \%$ glycerol. The prepared competent cells were then aliquoted into smaller volumes desired and stored in $80^{\circ} \mathrm{C}$ deep freezer before use.

### 2.9.2 Plasmid Transformation

Prepared DH5 $\alpha$ / B121 competent cells were first thawed on ice. 50 ng of plasmid DNA was added to the thawed competent cells $(20 \mu \mathrm{l})$ in a 1.5 ml microfuge tube and incubated on ice for 10 minutes. The cells / plasmid DNA mix were then subjected for incubation at $42^{\circ} \mathrm{C}$ for 45 sec and then immediately incubated on ice for 10 minutes. 1 ml of LB media were added to the mixture and the cells were recovered by incubation at 37 ${ }^{\circ} \mathrm{C}$ for an hour with shaking (200 rpm). $100 \mu \mathrm{l}$ of the transformed bacterial cells were then plated onto LB agar with selective antibiotics ( $100 \mu \mathrm{~g} / \mathrm{ml}$ of ampicillin or $30 \mu \mathrm{~g} / \mathrm{ml}$ of kanamycin). The LB agar was then subjected to $37{ }^{\circ} \mathrm{C}$ incubation for overnight. Positive transformation was identified by isolated bacterial colonies.

### 2.9.3 Glycerol Stock

An individual colony from the plasmid transformed bacterial plate was selected and inoculated into 5 ml of LB broth with the respective selective media. The bacteria culture was then incubated with shaking ( 250 rpm ) at $37^{\circ} \mathrm{C}$ overnight. $200 \mu \mathrm{l}$ of glycerol was added to $800 \mu \mathrm{l}$ of bacteria culture and mixed thoroughly. The glycerol stock was then labeled before storage into a $-80^{\circ} \mathrm{C}$ deep freezer.

### 2.9.4 Plasmid Amplification by QIAGEN QIAprep Miniprep

Plasmids of interest were transformed into $\mathrm{DH} 5 \alpha$ competent cells as discussed in the earlier section. An individual isolated colony was then selected and inoculated into 5 ml of LB broth with selective antibiotics. The culture was then incubated with shaking (250 rpm) at $37^{\circ} \mathrm{C}$ overnight. Overnight culture was subjected to centrifugation at 5,000 rpm for 10 min to pellet the bacteria cells. Supernatant was then decanted and the cells were resuspended thoroughly in $250 \mu$ l of ice-cold resuspension buffer, Buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, supplemented with $100 \mu \mathrm{~g} / \mathrm{ml}$ RNase A) and transferred to a 2 ml microfuge tube. $250 \mu \mathrm{l}$ of Lysis buffer P2 ( $200 \mathrm{mM} \mathrm{NaOH}, 1 \%$ SDS (w / v)) were then added and mixed by inversion 5-6 times. $350 \mu \mathrm{l}$ of Neutralization buffer, N3 (proprietary to QIAGEN) were then added and mixed immediately by inversion 5-6 times. The mixture were then subjected to centrifugation at $13,200 \mathrm{rpm}$ for 10 min . Supernatant containing the plasmid DNA was then applied to the QIAprep column supplied and subjected to centrifugation at $13,200 \mathrm{rpm}$ for 1 min for adsorption of plasmid DNA to the silica membrane. Plasmid DNA adsorbed were then subjected to a wash of $750 \mu \mathrm{l}$ Buffer PE (proprietary to QIAGEN) and incubated at room temperature for 5 min . The QIAprep column was then subjected to centrifugation at $13,200 \mathrm{rpm}$ for 1 min . After removal of the eluate, the column was subjected to a repeat round of centrifugation with the same conditions. The eluate container was then discarded and the upper portion of the QIAprep tube was transferred to a 1.5 ml microfuge tube. $30 \mu \mathrm{l}$ of elution buffer $\mathrm{EB}(10 \mathrm{mM}$ Tris- $\mathrm{Cl}, \mathrm{pH} 8.5)$ was added directly to the silica membrane of the QIAprep column to maximize yield and incubated for 1 min at
room temperature prior to centrifugation at $13,200 \mathrm{rpm}$ for 1 min . plasmid DNA collected as the eluate was subjected to quantification and stored at $-20^{\circ} \mathrm{C}$ before use.

### 2.9.5 Plasmid Amplification by QIAGEN Plasmid Midi Kit

As per QIAGEN QIAprep miniprep, transformed bacteria was inoculated into 5 ml of LB (with selective antibiotics added) and grown for 8 hours prior, to a 100 times scale up of the bacteria culture to a 100 ml volume in LB (with selective antibiotics added). The bacteria culture was then subjected to overnight incubation at $37{ }^{\circ} \mathrm{C}$ with shaking at 250 rpm . The next day, the bacteria cells were pelleted by centrifugation at $5,000 \mathrm{rpm}$ for 10 min at $4^{\circ} \mathrm{C}$. Supernatant was discarded and the pellet was resuspended in 4 ml of resuspension buffer, buffer P1 ( $(50 \mathrm{mM}$ Tris-Cl pH 8.0, 10 mM EDTA, supplemented with $100 \mu \mathrm{~g} / \mathrm{ml}$ RNase A). 4 ml of lysis buffer, P2 (200 mM NaOH, $1 \%$ SDS (w/v)) was then added and subjected to vigorous mixing by inversion for 5-6 times and incubated at R.T. for 25 min .4 ml of prechilled neutralization buffer, P3 (3 M potassium acetate pH 5.5 ) was then added and mixed by inversion for $5-6$ times to precipitate the proteins, genomic DNA and cell debris. The mixture was then incubated for 15 min on ice. Following the incubation, the mixture was then subjected to high speed centrifugation at $15,000 \mathrm{rpm}$ for 30 min at $4^{\circ} \mathrm{C}$. The supernatant containing the plasmid DNA was then decanted into a new centrifuge tube and centrifuged again at $15,000 \mathrm{rpm}$ for 15 min at $4{ }^{\circ} \mathrm{C}$. Concurrently, 4 ml of equilibration buffer, Buffer QBT ( 750 mM $\mathrm{NaCl}, 50 \mathrm{mM}$ MOPS pH 7.0, 15 \% isopropanol (v/v), $0.15 \%$ Triton X-100 (v/v)) was added into the QIAGEN-tip 100 and allowed to flow through the silica membrane of the tip by gravity. Supernatant from the above was then applied and allowed to flow through
completely for plasmid DNA adsorption to the membrane of the QIAGEN-tip 100. The adsorbed plasmid DNA was washed with 2 washes of 10 ml wash buffer, Buffer QC (1 $\mathrm{M} \mathrm{NaCl}, 50 \mathrm{mM}$ MOPS $\mathrm{pH} 7.0,15 \%$ isopropanol (v/v)) by gravity flow. Plasmid DNA was then eluted by addition of 5 ml of elution buffer, Buffer QF $(1.25 \mathrm{M} \mathrm{NaCl}, 50 \mathrm{mM}$ Tris-Cl pH 8.5, $15 \%$ isopropanol (v/v). Eluted DNA was then precipitated by adding 0.7 volumes worth of reagent grade isopropanol and centrifuged for $30 \mathrm{~min} 15,000 \mathrm{rpm}$ at $4^{\circ} \mathrm{C}$. Supernatant was then decanted with great care taken to retain the pellet. The pellet was then washed with 2 ml of $70 \%$ reagent grade ethanol by pipetting and centrifuged for a further $10 \mathrm{~min} 15,000 \mathrm{rpm}$ at $4^{\circ} \mathrm{C}$. Supernatant from this centrifugation was decanted carefully and the pellet was allowed to air dry for 15 minutes before resuspension in $100 \mu \mathrm{l}$ of TE buffer ( 10 mM Tris- $\mathrm{Cl} \mathrm{pH} 8.0,1 \mathrm{mM}$ EDTA)

### 2.9.6 Plasmid DNA Quantification by UV Spectrophotometer.

Plasmid DNA obtained by QIAprep miniprep or QIAGEN plasmid Midi kit was quantified by subjecting to UV spectrophotometry at $\mathrm{OD}^{260 \mathrm{~nm}}$ and $\mathrm{OD}^{280 \mathrm{~nm}}$ with a zero performed using the water for dilution. Essentially, the DNA was diluted $125 \mathrm{X}(e . g .2 \mu \mathrm{l}$ of plasmid DNA with $248 \mu \mathrm{l}$ of distilled water) and subjected to UV spectrophotometry. Plasmid DNA concentration was calculated by:
$\mathrm{OD}^{260 \mathrm{~nm}} \mathrm{X} 50 \mathrm{ng} / \mu \mathrm{l}$ X 125 (dilution factor) $=$ Concentration $\mathrm{ng} / \mu \mathrm{l}$
Purity of the plasmid DNA obtained was obtained by dividing $\mathrm{OD}^{260 \mathrm{~nm}}$ with $\mathrm{OD}^{280 \mathrm{~nm}}$ and a value between 1.7-1.9, would indicate a good preparation for the plasmid DNA.

### 2.9.7 Cloning of Gene of Interest

For cloning purpose, specific restriction enzyme digest sites to be used were determined in silico using VectorNTI software. Oligonucleotides encoding the selected restriction sites (which does not digest any portion of the gene of interest) were synthesized and used for amplification of the gene of interest by polymerase chain reaction (PCR). Typically, a PCR reaction composed of the pfu polymerase (1 unit, Promega), 10X pfu polymerase reaction buffer (added to 1 X ), forward ( $20 \mu \mathrm{M}$ ) and reverse primers $(20 \mu \mathrm{M})$, dNTP mix (10 nM each, Fermentas) and topped up with MilliQ water. PCR cycling conditions are usually an initial denaturation step at $95^{\circ} \mathrm{C}$ for 5 min followed by 30 cycles of (1) Denaturation at $95{ }^{\circ} \mathrm{C}$ for 1 min , (2) Annealing temperature $50-55^{\circ} \mathrm{C}$ for 2 min (depending on the length of the gene of interest, usually use of $1.5 \mathrm{~min} / \mathrm{Kb}$ DNA for $p f u$ polymerase), and (3) Extension temperature of $72{ }^{\circ} \mathrm{C}$. Following the PCR cycling, a final extension of $72^{\circ} \mathrm{C}$ for 10 min was usually performed and the PCR product was kept at $4{ }^{\circ} \mathrm{C}$ prior to usage.

### 2.9.8 Restriction Enzyme Digestion

New England Biolabs' restriction enzymes were used for all the cloning work. Basically, a set up of the following would be used: vector plasmid DNA and PCRamplified DNA of the gene of interest were added as template for the restriction digest reactions Individual restriction enzyme or dual restriction enzymes were made use of depending on the initially designed oligonucleotides for cloning. Enzymes are usually added in the concentration of 1 unit each. 100 X BSA supplied by NEB were added if
necessary for either of the restriction enzyme to work optimally. 10 X restriction enzyme buffer to be used for the reaction would be specific to the restriction enzyme used especially in the case of dual digestion reactions. Restriction enzyme digestion mix were then incubated at $37^{\circ} \mathrm{C}$ for at least 4 hr prior to gel extraction to purify the digested mix as well as to remove the excess restriction enzyme which might interfere with later ligation reactions. Calf intestinal alkaline phosphatase (CIAP) was added to the vector digestion mix and incubated for an additional $15-30 \mathrm{~min}$ at $37^{\circ} \mathrm{C}$ after restriction digestion. This step minimizes self ligation of the vector DNA especially in the case of use of single restriction enzyme site for cloning, by catalyzing the release of $5^{\prime}$ and $3^{\prime}$ 'phosphate groups.

### 2.9.9 Gel Extraction

Gel Extraction was performed using QIAquick gel extraction kit. Essentially, the restriction digest mix was subjected to a $0.8 \%$ agarose gel electrophoresis. The specific DNA of interest was excised to remove excess agarose and placed into a microfuge tube. Solubilization buffer, Buffer QG (proprietary to QIAGEN) was added in a 3 volume: 1 gel volume (weight / mg) of the excised agarose containing the DNA of interest. The mixture was then incubated for 10 min at $50^{\circ} \mathrm{C}$ for the melting of the agarose. To ensure proper melting, the microfuge tube was subjected to pulse vortexing every $2-3 \mathrm{~min}$. 1 gel volume of isopropanol was then added and the mixture was applied to a QIAquick spin column. DNA was bound to the silica membrane by centrifugation at $13,200 \mathrm{rpm}$ for 1 min . Flow through was discarded and the collection tube was reused. $500 \mu \mathrm{l}$ of buffer QG was applied and centrifuged for 1 min at $13,200 \mathrm{rpm}$ to ensure removal of trace
amounts of agarose. The flow through was decanted and the collection tube was reused. $750 \mu \mathrm{l}$ of wash buffer, Buffer PE was then applied to the QIAquick spin column and allowed to incubate at R.T. for 5 min. It was then subjected to centrifugation at 13,200 rpm for 1 min . A repeat centrifuge was performed to ensure removal of all traces of wash buffer. QIAquick spin column was transferred to a new 1.5 ml microfuge tube and appropriate amount of elution buffer, Buffer EB (10mM Tris-Cl, pH 8.5) was applied and incubated for a further 5 min prior to centrifugation at 13,200 rpm for 1 min . The eluate from the centrifugation contains the purified DNA.

### 2.9.10 Ligation Reaction

For ligation of the restriction digested plasmid and gene of interest, T4 DNA ligase (NEB) were made use of. The mixture essentially comprises of 8 : 1 concentration ratio between gene of interest to vector, 10 X T4 DNA ligase buffer (made up to 1X, NEB), T4 DNA ligase (1 unit) and minimal amount of distilled water. Ligation reactions were then incubated at $16{ }^{\circ} \mathrm{C}$ overnight or $4^{\circ} \mathrm{C}$ for 8 hr , prior to transformation for positive clones. Colonies were subjected to colony PCR and / or plasmid extraction followed by restriction enzyme digest to confirm the positive cloning of the gene. Also, all plasmid were subjected to sequencing using specifically designed oligonucleotides upstream and downstream of the cloning sites to ensure no mutations have occurred during the entire process of cloning.

### 2.9.11 DNA Sequencing

Sequencing was performed by National Neuroscience Institute (NNI) Core Facility, Tan Tock Seng Hospital (TTSH). Selective sequencing primers were designed $100-200 \mathrm{bp}$ upstream of the site of interest. Briefly, 500 ng of plasmid DNA were added into a 0.2 ml PCR tube followed by addition of 1 mM of specifically designed sequencing primers (usually designed by use of software about $100-150 \mathrm{bp}$ upstream of the sequence of interest). Sequencing was carried out and upon completion, sequencing data $\begin{array}{lllll}\text { were analyzed using } & \text { freeware, } & \text { Chromas } & 1.45\end{array}$ (http://www.technelysium.com.au/chromas14x.html), which allows the export of the text data for further analysis using VectorNTI suite of software.

## Chapter 3 Proteome Profiles of HBx Genotypes

Carriers of distinct genotypes of HBV have resulted in differing disease progression as highlighted by Kao et. al., 2002a and Schaefer et. al., 2005. As such, it would be interesting to clone replicative HBV genomes (rHBV) of these genotypes and their viral protein(s) and understand potential differential effects they might have on the host cells. On the other hand, the viral protein HBx is a major causative agent previously studied to have transforming potential and included in this part of the study. HBV genotypes B and C present more severe forms of liver diseases in HBV carriers, such as liver cirrhosis and hepatocellular carcinoma (Arbuthnot and Kew, 2001) and is endemic in the Asia region (Figure 1.4) where more people are suffering as carriers of HBV (Table 1.1).

### 3.1 Database Search and Multiple Sequence Alignment

Sequences of HBV genotypes B and C were downloaded and aligned for sequence comparison. Conserved sequences were then used for the design of specific primers for the entire genome or a particular gene. Entire genetic sequences of HBV virus, of genotype B and C respectively, were downloaded from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The sequences for HBV virus genotype B (accession nos. AB106884, AB106885, AY167100, AY167101, AY206387, AY206390 and AY206391) and genotype C (accession nos. AB033551, AB033553, AB112065, AB112066, AB112348, AB112471, AB112472, AF330110, AF411412, AF533983, AY206392 and AY306136) were used. In addition to the minute differences in genetic sequences within the same genotype (See Appendix Fig 9.1), distinct differences between the sequences of genotype B and C were observed,
particularly within the Surface region between 2874-833bp. Overall, the difference was approximately $9.4 \%$ between the genotype B and C and this result was in line with earlier reports (Okamoto et. al., 1988) suggesting more than $8 \%$ differences between genotypes.

### 3.2 Genotyping of Genotypes B and C

The entire HBV viral Surface gene (1174bp) were first amplified from HBV viral DNA (extracted from clinical samples using QIAGEN Blood mini kit) by pfu polymerase as observed in figure 3.1 (A). DNA sequencing was performed using a specifically designed internal primer for the surface antigen starting from position 155bp (Start of the smallest Surface gene). Sequences obtained were then subjected to analysis by VectorNTI detecting specific restriction sites for genotyping as previously reported (modified from RFLP method by Mizokami et. al., 1999). Specific restriction sites analyzed in silico were determined to be AlwI, NlaIV, EarI and HphI. Absence of EarI sequence 83 bp downstream of conserved $H p h I$ as well as presence of $A l w I$, signifies a genotype B while those with a EarI site were of genotype C (Refer to Figure 3.1(B) for graphical representation). Samples with confirmed genotypes were further used for cloning of replicative HBV genome of each genotype as well as their respective HBx into mammalian and bacterial expression vectors.


Figure 3.1:
Genotyping of HBV genomes from clinical samples
(A) PCR of entire Surface antigen (1174bp) for sequencing. Not all clinical samples can be amplified by the same primer set. Lanes 2 and 4 signifies PCR amplification from HBV viral DNA extracted while lanes 1 and 6 represents the negative controls for the PCR reaction using distilled water as template. Red arrow points to the amplified products. The product was subjected to sequencing using internal designed primers.
(B) Restriction Map created in silico by VectorNTI after sequencing of PCR products for Surface Antigen from HBV viral DNA (Figure 3.1 panel A). Various restriction enzymes namely AlwI, HphI, Earl and NlaIV were used for genotyping. In particular, Genotype $B$ was distinguished by the presence of AlwI (boxed in blue) while Genotype $C$ was differentiated by the presence of EarI 83 bp downstream of conserved HphI between the 2 genotypes. This is a modified method from physical usage of restriction enzymes to identify RFLP in the various genotypes (Mizokami et. al., 1999)

### 3.3 Cloning of Replicative HBV Genomes of Specific Genotypes and their Respective HBx

### 3.3.1 HBV Replicative Genome (rHBV) Cloning

Replicative genomes consisting of $\sim 1.1 \mathrm{X} \mathrm{HBV}$ genome in length with terminal redundancy were constructed to reflect the in vivo infection or replication environment, whereby the pregenome RNA is the template for HBV replication (Weiss et. al., 1996; Chen et. al., 2000). PCR amplification was performed by pfu polymerase (Promega) to obtain the 2 fragments for ligation to construct the replicative HBV genome. Hence, the cloning of the HBV genome will require the ligation of 2 fragments to generate terminal redundancy. The region 1600-1900 bp of HBV virus, comprising both the DR1 and DR2 (direct repeats) sequences necessary for HBV replication (Refer to Figure 1.2), flanks the entire HBV sequence in a continuous fashion. Primers for replicative HBV genome cloning were designed based on the conserved regions and unique restriction sites shared by both HBV genotypes. In particular, MluI was used for cloning of the entire 1.1 X HBV genomes into mammalian expression vector, pCDNA3.1+ and a conserved internal XbaI (within the Surface gene) restriction site was used for internal cloning. Strategy for construction is attached at Appendix Figure 9.2.

As XbaI serves as the unique restriction site within HBV genome, it was chosen for cloning. Therefore, the XbaI from the multiple cloning site of pCDNA3.1+ was removed first by digestion with XbaI followed by Klenow fragment treatment and finally re-ligation with T4 DNA ligase. The mutated vector was confirmed for removal of XbaI site by 2 single digestion using $M l u I$ and $X b a I$ respectively to obtain linearized and uncut
plasmid respectively. The mutation was further confirmed by sequencing prior to being used for subsequent cloning.

PCR products of the 2 fragments were first TOPO-cloned (Refer to Figure 3.2 panelA) into Zero Blunt TOPO PCR kit (Invitrogen Inc., USA) due to difficulty in obtaining sufficient amounts of clinical viral HBV DNA for direct cloning as well as to minimize the danger of handling infectious materials. After plasmid amplification, the fragments were excised with respective enzymes, MluI ( $1^{\text {st }}$ fragment $\sim 1.9 \mathrm{~Kb}$ ) and XbaI ( $2^{\text {nd }}$ fragment $\sim 1.6 \mathrm{~Kb}$ ), and cloned sequentially into XbaI-removed pCDNA3.1+ (Figure 3.2 panel A)

TOPO-cloned fragment, 1600bp to 248 bp (of respective genomes), was digested by flanking MluI enzyme, gel-purified and ligated to pCDNA3.1+ (previously treated to remove $X b a I$ ) at the MluI site. As designed, this fragment carries an internal XbaI site at the 3' end which allows for the ligation of fragment 2, flanked by XbaI sites. This ligation will complete the HBV genome and join the 2 fragments at the HBV conserved XbaI site which corresponds to position 248bp (Appendix Figure 9.1, boxed Fragment $25^{\prime}$ end).

Cloning of the replicative genomes of specific genotype B and C HBV have been established. A representative digestion confirmation of successful cloning was depicted in Figure 3.2 (B). Interestingly, one of our successfully cloned Genotype B replicative HBV genome consists of a previously unidentified 102 bp in-frame deletion (Appendix Figure 9.3) in the C-terminal of the PreS1 to N-terminal of PreS2 segment of the viral
(A)

(B)


Figure 3.2:
Cloning of replicative HBV genomes
(A) TOPO cloning of $1^{\text {st }}$ and $2^{\text {nd }}$ fragments for replicative HBV genome construction. Lane 1 represents MluI digested TOPO cloned fragment 1 ( $\sim 1.9 \mathrm{~Kb}$ ). Lane 2 signifies XbaI digestion of TOPO cloned fragment $2(\sim 1.6 \mathrm{~Kb})$. These fragments will then be gel-purified and cloned into pCDNA3.1+.
(B) Example of the cloning of rHBV into pCDNA3.1+. (Refer to cloning strategy in Appendix Figure 9.2) Essentially, the cloning took two steps. First, the cloning of the first fragment which consist the internal XbaI digestion site for insertion of the second fragment without frame shift. This figure shows the correct digestion profile of the designed cloning strategy. MluI was used to digest out the entire $3.5 \mathrm{~Kb} \mathbf{~ r H B V}$ genome (Lane 2) while XbaI digestion yielded only the second fragment (Lane 3). Lane 1 shows the undigested pCDNA3.1+rHBV genome.
genome. Other mutations in the surface antigen coding region have been reported (Gallina et. al., 1995, Xu et. al., 1996, Chen et. al., 2002) and have been established as potential liver disease causative factor due to the retention of surface antigen in the hepatocytes. It would hence be interesting to show how this mutation affects HBV carriers, as well as conduct clinical screens on the prevalence of such mutants in HBV carriers.

### 3.3.2 Confirmation of Positive Cloning of HBV Genome.

Initially, genotype A rHBV has been established in our lab (Chen et. al., 2000a). Making use of this construct in the transient transfection of various liver cell lines, HepG2 (ATCC, USA) and Chang liver cells (ATCC, USA), we showed the presence of HBsAg production using Abbott Laboratories' IMX MEIA analyses utilizing antibodies against surface antigen coupled to an enzyme immunoassay (Figure 3.3 panel A). This analytical machine has been used in hospital for the screening of patient's serum for HBsAg and is hence very specific. Surprisingly, rHBV transfection into HEK293T cells also showed high HBsAg production (Figure 3.3 panel A). This could be explained by the fact that we are introducing the plasmid by transfection into the cells serving as template for viral replication compared to natural state, where the virus has to select specific cell types and enter via receptor ligand interaction. The high level of HBsAg production in HEK293T cells could be attributed to its high transfection efficiency compared to the two liver cell lines. Controls provided by the kit consists of the positive control which shows excessively high levels of HBsAg due to the concentrate as well as a negative control containing no HBsAg and serve as the extreme ends of the test. Therefore, we have
established a procedure to test for Hepatitis B virus surface antigen (HBsAg) production and HEK293T could be used in later works for rHBV transfections.

All three genotypes of rHBV (Genotype A as previously constructed, Genotype B and C ) showed positive HBsAg production with genotype A producing more HBsAg particles compared to genotypes B and C when transiently transfected into Chang liver cells (Figure 3.3 panel B). The high levels of positive control and negative control was expected due to purified HBsAg used by the kit and our transfection negative control using empty vector showed no reactiveness. To date, the rHBV of these genotypes constructed have been used by our lab to analyze for the production of Hepatitis B virus splice protein (HBSP) (Soussan et. al., 2003, Lu et. al., 2006) as well as their potential implication in apoptosis.

As observed in Figure 3.4 (A), rHBV of the various genotypes transfected into HepG2 cells showed production of HBSP using Western analysis on protein lysates after 48 hours post transfection (lanes 4, 5 and 6 corresponds to protein lysates from rHBV genotype A, B and C transfected HepG2 cells respectively). Also, since HBSP is a secretable protein, it was also detected in the spent media after 48 hours by concentration of the protein (lanes 1, 2 and 3 corresponds to media protein Western from rHBV genotype A, B and C transfected HepG2 cells respectively). This work and the apoptotic related work below was performed by a fellow laboratory member, Lu Yiwei after my treatment of the cells.
(A)

(B)


Figure 3.3:
(A) IMX Analysis for HBsAg production from rHBV genotype A transfected intro various cell lines. Cutoff point for positive and negative results are indicated by the red dashed horizontal line (average of rate $=14$ ) as determined by the calibration. As observed, rHBV transient transfection gave positive albeit low rate for HepG2 and Chang liver cells. However, HEK293T showed surprisingly high HBsAg rate. This could be attributed to the fact that HEK293T can be transfected more efficiently compared to the other 2 cell lines (HEK293T efficiency was measured through the transfection of pEGFP-C1 (Clontech) and measuring \% of transfected cells against total cell number. Results obtained showed about $85 \%$ green cells.
(B) IMX Analysis of $\mathbf{r H B V}$ of Genotype $A, B$ and $C$ compared to empty vector, pCDNA3.1+ transiently transfected into Chang liver cells. Red dashed horizontal line (average of rate $=8.6$ ) as determined by calibration serve as the cut off point between reactive and non-reactive for HBsAg production. As observed, genotype A rHBV produces the most HBsAg detected as compared to genotypes of $B$ and $C$ rHBV.

Previously, it has been shown that HBx and HBSP of the HBV, can cause apoptosis (Lu and Chen, 2005, Lu et. al., 2006). Therefore, we subjected the rHBV of genotypes A, B and C to analyses of their apoptotic effect on HepG2 cells transiently transfected. Briefly, HepG2 cells were nucleofected (Amaxa, Gmbh) by the various genotypes of rHBV as well as pCDNA3.1+ (acting as empty vector). 24 hours and 48 hours post transfection, cells were collected respectively. Flow Cytometry was used for this analysis using AnnexinV - FITC (One of the earliest presentation of apoptosis is the externalization of the phospholipids phosphatidylserine (PS) from inside to the outside environment. AnnexinV has high affinity for phospholipids especially towards PS. Using FITC as the tag, staining indicates apoptotic cells) and Propidium Iodide (PI) to double stain the apoptotic cells (PI is a membrane impermeant stain dye, intercalating DNA nonspecifically. Therefore, presence of staining could be used to show loss of membrane integrity which occurs during cellular apoptosis) and the results are charted in Figure 3.4 (B). Briefly, the lower right panel of each chart A to H shows the amount of apoptotic cells. Negative control showing basal level of apoptosis was obtained by transfection of pCDNA3.1+ (panel A). Positive control showing high apoptotic level was obtained by HepG2 cells treatment with Cisplatin (panel B), a known inducer of cellular apoptosis (Cisplatin is a platinum based chemotherapeutic drug and known to cause cell death). Panels C, D and E (24 hr) and Panels F, G and H (48 hr) corresponds to the rHBV genotype transfected $A, B$ and $C$ for the respective number of hours. As tabulated, genotype B shows the highest apoptosis compared to genotype A and C rHBV transfected HepG2 cells. The difference was more significant 48 hours post-transfection
(A)

(B)

A


F

G

H


Figure 3.4:
Functional analysis of the constructed rHBV genomes A, B and C.
(A) Replicative genome A, B and C were transiently transfected into HepG2 cells and incubated for 48 hours. Proteins were extracted from the cells and probed with antiHBSP in conjunction with those in the media. Lanes 1, 2 and 3 shows HBSP production in the spent culture media of genotype $A, B$ and $C$ transfection respectively. Lanes 4,5 and 6 shows the HBSP production intracellularly of the same transfection.
(B) Flow Cytometry analysis of apoptotic effect by HBV genome A, B, and C. HepG2 cells were transiently transfected with HBV genome A, B, and C and incubated for 24 h (panel C, D, E, respectively) and 48 h (panel F, G, H, respectively). Cells were collected respectively and applied to FACs assay. Cells transfected with empty vector pcDNA3.1 (panel A) and cells treated with cisplatin (panel B) serves as the corresponding negative and positive controls. AnnexinV-FITC (FL1-H) and Propidium Iodide (FL2-H) was used to double stain the cells.

In each panel, the lower right square indicates the number of apoptotic cells.
Data: A:0.62\%, B: $\mathbf{1 7 . 3 2 \%}$, C: 2.53\%, D: $2.69 \%$, E:1.61\%; F:3.76\%, G: 6.64\%, H: 2.82\%
and showed approximately 2 fold increase in the number of apoptotic cells (B (48 hr) $6.64 \%)$ compared to the other two genotypes ( $\mathrm{A}(48 \mathrm{hr})-3.76 \%, \mathrm{C}(48 \mathrm{hr})-2.82 \%)$.

Therefore, it seems that the rHBV cloned showed similar HBsAg detectable and also possesses the HBSP fragment. Hence, further characterization of the apoptotic effects observed might allow us to gain some useful insights as to how HBV of various genotypes might be different. Work is ongoing toward this goal.

### 3.3.3 HBx Cloning

Based on its interactions with the wide range of cellular proteins (Bouchard and Schneider, 2004) and its potential role in the development of HCC, HBx has been included into our study. Interestingly, variability in genetic sequences of such conserved and compact protein has been observed (Figure 3.5) between the HBx from database sequences of the various genotypes translated in silico using VectorNTI software. Therefore, it is possible that such differences between HBx of variant genotypes may play a role in the differential disease progression observed clinically since HBx have been implicated as a determinant in liver disease outcome. Interestingly, the major differences were localized in a region with proline-rich sequences.

HBX ORF sequences (1374bp - 1836bp) were also aligned based on the above sequences for primer design in cloning of HBX into mammalian expression vector, pCDNA3.1+ (Invitrogen Inc., USA). HBx of genotypes B and C, were first cloned into pCDNA3.1+ using unique restriction sites EcoRV and NotI determined by sequence

Genotype A HBX
Genotype B HBX
Genotype C HBX
Genotype D HBX
Genotype E HBX
Genotype F HBX
Genotype G HBX
Genotype H HBX

Genotype A HBX
Genotype B HBX
Genotype C HBX
Genotype D HBX
Genotype E HBX
Genotype $F$ HBX
Genotype G HBX
Genotype H HBX

Genotype A HBX
Genotype B HBX
Genotype C HBX
Genotype D HBX
Genotype E HBX
Genotype $F$ HBX
Genotype G HBX
Genotype H HBX

Genotype A HBX
Genotype B HBX
Genotype C HBX
Genotype D HBX
Genotype E HBX
Genotype F HBX
Genotype G HBX
Genotype H HBX

1
50
(1) AARLYCQLDPSRDVLCLRPVGAESRGRPLAGPLGTLSSPSPSAVPADHG
(1) AARLCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPASPPIVPTDHG
(1) AARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGTLPSPSSSAVPADHG
(1) AARLCCQLDPARDVLCLRPVGAESRGRPFSGPLGTLSSPSPSAVSSDHG
(1) AARLCCQLDPARDVLCLRPVGAESCGRPVSGSLGGLSSPSPSAVPADHG
(1) AARMCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPSSASAVPADHG
(1) AARLCCQLDPTRDVLCLRPVGAESSGRSLSGSLGAVSPPSPSAVPADDG
(1) AARLCCQLDPARDVLCLRPVGAESCGRPLSWSPGALPPPSPPSVPADDG 51100
(51) HLSLRGLPVCAFSSAGPCALRFTSARCMETTVNAHQILPKVLHKRTLGL
(51) HLSLRGLPVCAFSSAGPCALRFTSARRMETTVNAHRNLPKVLHKRTLGL
(51) HLSLRGLPVCAFSSAGPCALRFTSARRMETTVNARQVLPKVLHKRTLGL
(51) HLSLRGLPVCAFSSAGPCALRFTSARRMETTVNAHHFLPKVLHKRTLGL
(51) HLSLRGLPVCAFSSAGPCALRFTSARRMETTVNAHQILPKVLHKRTLGL
(51) TSSLRGLPVCSFSSAGPCALRFTSARRMETTVNAPWSLPTVLHKRTIGL
(51) HLSLRGLPVCSFSSAGPCALRFTSARRMETTVNAPRSLPTVLHKRTLGL
(51) HLSLRGLPACAFSSAGPCALRFTSARRMETTVNAPQSLPTTLHKRTLGL 101

150
(101) PAMSTTDLEAYFKDCVFKDWEELGEEIRLKVFVLGGCRHKLVCAPAPCNF
(101) SAMSTTDLEAYFKDCVFNEWEELGEEIRLKVFVLGGCRHKLVCSPAPCNF
(101) SAMSTTDLEAYFKDCVFTDWEELGEETRLKIFVLGGCRHKLVCSPAPCNF
(101) SVMSTTDLEAYFKDCLFKDWEELGEETRLKVFVLGGCRHKLVCAPAPCNF
(101) SAMSTTDLEAYFKDCLFKDWEELGEEIRLKVFVLGGCRHKLVCVPAPCNF
(101) SGRSMTWIEDYIKDCVFKDWEELGEEIRLKVFVLGGCRHKLVCSPAPCNF
(101) SGRSITWIEEYIKDCVFKDWEELGEEIRLMIFVLGGCRHKLVCSPAPCNF
(101)SPRSTTWIEEYIKDCVFKDWEESGEELRLKVFVLGGCRHKLVCSPAPCNF 151
(151) FTSA
(151) FTSA
(151) FTSA
(151) FTSA
(151) FTSA
(151) FTSA
(151) FTSA
(151) FTSA

## Figure 3.5:

Alignment of the amino acid sequence of a representative of HBx sample from each genotype of HBV from database sequences. Differences can be observed throughout the HBx amino acid sequences particularly just before the amino acid 50.

## Color Coding

(1) Yellow : Completely conserved residue at given position.
(2) Blue : Consensus residue derived from a block of similar residues at a given position.
(3) Green : Residue weakly similar to consensus residue at given position.
(4) Black letterings : Non-similar residues.

```
Genotype B(HBX) 1 MAARLCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPASPPIVPTDHG
Genotype C(HBX) 1 MAARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGTLPSPSSSAVPADHG
Genotype B(HBX) 51 AHLSLRGLPVCAFSSAGPCALRFTSARRMETTVNAHRNLPKVLHKRTLGL
Genotype C(HBX) 51 AHLSLRGLPVCAFSSAGPCALRFTSARRMETTVNARQVLPKVLHKRTLGL
Genotype B(HBX)101 SAMSTTDLEAYFKDCVFNEWEELGEEIRLKVFVLGGCRHKLVCSPAPCNF
Genotype C(HBX)101 SAMSTTDLEAYFKDCVFTDWEELGEETRLKIFVLGGCRHKLVCSPAPCNF
```

Genotype B(HBX) 151 FTSA
Genotype C(HBX) 151 FTSA

## Figure 3.6:

HBx from clinical samples were cloned into pCDNA3.1+ and sequenced. In silico translation into amino acids was performed with VectorNTI. Note that most of the HBX sequences are conserved with majority of amino acid differences between 3143bp and 86-88 are highlighted in blue. In a relatively small viral protein HBx, the differences could be important in differentiating its downstream outcome. Color Coding
(1) Yellow : Completely conserved residue at given position.
(2) Blue : Consensus residue derived from a block of similar residues at a given position.
(3) Green : Residue weakly similar to consensus residue at given position.
(4) Black letterings : Non-similar residues.
analyses. Cloned HBx were sequenced and translated in silico into their respective amino acid sequences (Figure 3.6). Sequences are relatively conserved (91\%) but noticeably, there are two particular regions of differences between the 2 HBV genotypes. The major differences can be observed in: (a) Between amino acids 31-43, more proline residues were found in genotype $B$ in contrast to serine residues in genotype $C$. This could possibly cause a structural and functional difference between the viral HBx proteins of different genotypes; (b) at the region 86-88 bp, we observed a difference in a triad of amino acid residues namely His-Arg-Asn to Arg-Gln-Val. These two major differences are also conserved in database sequences as observed in an alignment of the cloned sequence with a representative database sequence (AY167101 for Genotype B and AB033551 for Genotype C) from each genotype (Figure 3.7 panel A) and hence strengthen our hypothesis that they could potentially result in a difference in structure and functions between HBx of genotypes B and C . Of interest, proline rich sequences are often implicated in cellular signaling, through the binding to SH3 domain-containing cellular proteins (Nguyen et. al., 1998).

### 3.3.4 Expression of Cloned HBx of the Various Genotypes.

Cloned HBx of genotypes A, B and C as well as the empty plasmid, pCDNA3.1+ were transiently transfected into Chang liver cells and their expression was examined. Antibodies for HBx have been elusive and recent studies have shown that differences in epitopes (due to differences in genotypes of HBx ) have made it difficult for development of HBx antibodies. Pál et. al., 2003, mentioned the need for both the use of complex immunological as well as current biological techniques for mapping of the proper epitope.
(A)

```
B_AY167101_HBX
Genotype B HBX
C_AB033551_HBX
Genotype C HBX
B_AY167101_HBX
Genotype B HBX
C_AB033551_HBX
Genotype C HBX
B_AY167101_HBX
Genotype B HBX
C_AB033551_HBX
Genotype C HBX
B_AY167101_HBX
Genotype B HBX
C_AB033551_HBX
Genotype C HBX
```

1
(1) MAARLCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPASPPVVPTD
(1) MAARLCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPASPPIVPTD
(1) MAARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGPLPSPSSSAVPAA
(1) MAARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGTLPSPSSSAVPAD 49

96
HGAHLSLRGLPVCAF SSAGPCALRFTSARRMETTVNAHRNLPKVLHKR
HGAHLSLRGLPVCAFSSAGPCALRFTSARRMETTVNAHRNLPKVLHKR
HGAHLSLRGLPVCAFSSAGPCALRFTSARRMETTVNTHQVLPKVLHKR
HGAHLSLRGLPVCAFSSAGPCALRFTSARRMETTVNARQVLPKVLHKR 97144
TLGLSAMSTTDLEAYFKDCVFTEWEELGEEIRLKVFVLGGCRHKLVCS
TLGLSAMSTTDLEAYFKDCVFNEWEELGEEIRLKVFVLGGCRHKLVCS
TLGLSAMSTTDLEAYFKDCLFKDWEELGEEIRLKVFVLGGCRHKLVCS
TLGLSAMSTTDLEAYFKDCVFTDWEELGEETRLKIFVLGGCRHKLVCS 145154
PAPCNFFTSA
PAPCNFFTSA
PAPCNFFTSA
PAPCNFFTSA
(B)


Figure 3.7:
Conserved sequences amongst genotypes and HBx expression
(A) Sequence alignment of Cloned $H B x$ of the two genotypes $B$ and $C$ showed close alignment with representative database sequence of their respective genotypes (B_AY167101 and C_AB033551). This shows the mutations occurring in clinical samples were conserved within genotypes. Please refer to Figure 3.5 \& 3.6 for color coding legend.
(B) RT-PCR of transfected cloned HBX into Chang Liver cells. Lanes 1, 2 and 3 corresponds to Chang liver cells transfected with genotypes A (previously cloned), B and C HBX. Lane $M$ depicts the 1 Kb DNA ladder used (Fermentas) while lane 4 depicts Chang cells transfected with empty vector, pCDNA3.1+.

As suitable antibodies were unavailable commercially, we made use of RT-PCR (QIAGEN One-Step RT-PCR kit) using specific HBx primers on the total RNA extracted from transfected Chang liver cells, after treatment with DNaseI to remove genomic and plasmid DNA interference (Chen et. al., 2000b). As seen in Figure 3.7 (B). mRNA production were observed for cells transfected with all genotypes of HBx but not in those transfected with the empty vector pCDNA3.1+. This indicated positive HBx mRNA production and expression.

In parallel, the same HBx sequences were cloned into pEGFP-C1 to generate GFP-fusion proteins with the Green Fluorescent Protein (GFP) to allow for visualization of HBx localization (Chen et. al., 2001b). As previously studied, it was believed that HBx was localized in the proteasome. Also, it was observed that HBx was localized in the peri-nuclear region. As observed in Figure 3.8 (A) GFP-fusion constructs of HBx was transiently transfected into HepG2 cells and protein was extracted 48 hr post-transfection for Western analysis using antibodies targeted for GFP (Santa Cruz Biotech). Proper expression of the GFP-fusion proteins was observed in lane 1 (GFP vector only transfection) with an expected size at $\sim 26 \mathrm{kDa}$. Lanes 2 and 3 showed HBx fusion construct with molecular size of $\sim 43 \mathrm{kDa}$ which was correct as HBx codes for $\mathrm{a} \sim 17 \mathrm{kDa}$ protein. These constructs were next transfected transiently into liver cell line, Huh7 cells to examine their localization. As observed in Figure 3.8, HBx of genotypes B (Figure 3.8 panel B) and C (Figure 3.8 panel C) did not show much difference in localization between each other. They were both located at the nuclear periphery with punctuated staining as previously reported (Chen et. al., 2001b). Therefore, the difference in the
(A)

(ii)
(iii)
(B)

(i)
(ii)

(i)
(ii)
(iii)
(D)


Figure 3.8:
Expression of pEGFP-HBx FL construct and localization.
(A) pEGFP-C1 cloning of HBx FL of genotypes B and C. $40 \mu \mathrm{~g}$ of cell lysates from untransfected or transfected HepG2 cells were used per sample. Lane 1 depicts the vector (pEGFP-C1) alone transfected HepG2 cell lysates. Lanes 2 and 3 depicts the pEGFP-C1 HBx B FL and HBx C FL transfected HepG2 cell lysates respectively. Lane 4 shows the untransfected HepG2 cell lysates. Western analysis of the pEGFPC1 constructs using Polyclonal Rabbit anti-GFP antibodies (Santa Cruz Biotech). The expression of GFP (MW of 26 kDa ) was observed clearly in the empty vector transfected cells. Lanes 2 and 3 showed the expression of pEGFP-C1 HBx B FL and pEGFP-C1 HBx C FL at MW of $\sim 43 \mathrm{kDa}$ respectively compared to vector alone. Lane 4 presented a non-specific band at MW > 45 kDa as seen in the other lysates, a possibility that this antibody reacts with intrinsic HepG2 proteins.
(B) Transient Transfection of empty vector, pEGFP-C1 (Clontech) into HepG2 cells. Cells were fixed with 3 \% Paraformaldehyde and then mounted with DAPI staining for the nucleus (Molecular Probe). (i) depicts the DAPI stained nucleus while (ii) shows the expression of GFP. Images were overlayed using Adobe Photoshop CS and depicted in (iii). As expected, GFP showed no particular area of localization.
(C) Transient Transfection of pEGFP-C1-HBx Genotype B into HepG2 cells. Cells were fixed with $3 \%$ Paraformaldehyde and then mounted with DAPI staining for the nucleus (Molecular Probe). (i) depicts the DAPI stained nucleus while (ii) shows the expression of GFP. Images were overlayed using Adobe Photoshop CS and depicted in (iii). HBx showed a specific localization around the periphery of the nucleus. This has been previously shown in genotype A HBx localization in contrast with empty vector transfection.
(D) Transient Transfection of pEGFP-C1-HBx Genotype C into HepG2 cells. Cells were fixed with 3 \% Paraformaldehyde and then mounted with DAPI staining for the nucleus (Invitrogen Inc., USA). (i) depicts the DAPI stained nucleus while (ii) shows the expression of GFP. Image was overlayed using Adobe Photoshop CS and depicted in (iii). HBx showed similar localization to that of genotype $B$ and previously reported genotype $A$ in contrast with empty vector transfection.

White line signifies $50 \boldsymbol{\mu m}$.
amino acid sequence did not have any impact on cellular localization. To observe a global difference, HBx cloned into pCDNA3.1+ of genotypes B and C were transfected into Chang liver cells (ATCC, USA) and subjected to 2 Dimension electrophoresis to understand if they cause a difference in proteome expression.

### 3.4 Two-Dimensional Electrophoresis \& Spot Identification

2DE was performed on total proteins extracted from Chang Liver cells transfected with the HBx constructs. HBx of specific genotypes were first used for proteome profiling. Comparisons were made between HBx-genotype B , HBx-genotype C and pCDNA3.1+ (vector only-negative control) transfected cells. At least three independent 2D-PAGE runs (as described, Sections 2.4) were performed per sample, to eliminate potential gel-to-gel variations. Typically, a confluent flask ( $75 \mathrm{~cm}^{2}$ ) of cells yields approximately $500-700 \mu \mathrm{~g}$ of total proteins. $80 \mu \mathrm{~g}$ of extracted total proteins were used for each analytical gel run and $200 \mu \mathrm{~g}$ were used for the eventual preparative gel destined for Mass-Spectrometry.

Initially, the analytical gel pictures were identified visually to denote spots which show major differences. Differences in protein spots between gels were charted based on their presence or absence on all 3 gels. Ambiguous differences (presence or absence of spots not consistent) were also noted. Figure 3.9 shows a general overview of the entire gel run compared between HBx genotype B and C transfected to pCDNA3.1+ transfected HepG2 cells. The overall profile appeared to be identical and some proteins were selected as landmarks for identification of differential expressing proteins. They were further


Figure 3.9:
2-dimension SDS-PAGE gel of total proteins extracted from ATCC-Chang Liver cells after transfection with (A) Empty plasmid (pCDNA3.1+ only), (B) HBX Genotype B transfected and (C) HBX Genotype C. Specific areas of interest are boxed in (a) and are magnified in Figure 3.9.

## Differences

pCDNA3.1+
HBX_B


15


Figure 3.10:
Magnification of various differences (grouped into A-G) noted visually on comparison of the empty plasmid (pCDNA3.1+ only) transfected, HBx_Genotype B and HBx_Genotype C. Specific spots related to Genotype B HBX are boxed in blue while those related to Genotype $C$ are boxed in red. Common differences were boxed in green
analyzed based on their molecular weight and $p I$ values. Noticeable differences were identified between pCDNA3.1+ transfected gel and the HBx transfected ones. The gels were further dissected (for ease of reading) into regions as boxed in Figure 3.9, and magnified in Figure 3.10 in terms of gel regions A-G, in conjunction with each other.

The estimated $p I$ values and molecular weight ( kDa ) of these different proteins were assessed and summarized in Table 3.1. Results indicated four categories of differences:
(a) Proteins not present in pCDNA3.1+ transfected (negative control) Chang liver cells, but present in cells transfected with HBx of either genotypes B and C. These includes spots 1, 2, 5, 9 and 11;
(b) Protein which was present in control transfected Chang liver cells but not present in both genotype transfected cells. These include spot 4;
(c) Proteins which were not present in control but present only in Genotype B transfected Chang liver cells. These include spots $3,7,12,13,14$ and 15 ;
(d) Proteins which were not present in control but present only in Genotype C transfected Chang liver cells. These include spots 6, 8, 10 and 16.

As collated above, certain differences were found to be associated with exposure to particular genotype of HBx , while others were just different between HBx transfection and empty vector transfection. Some spots were selected to be further analyzed by Matrix-Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometry

Table 3.1: Isoelectric Points ( $p I$ ) and Molecular Weight (M.W.) values of cellular proteins associated with Genotype Specific HBx.

| Spot <br> No. | Approximate pl | Approximate Molecular <br> Weight (M.W.) kDa | HBx <br> Genotype <br> B-specific | HBx <br> Genotype <br> C-specific |
| :--- | :---: | :---: | :---: | :---: |
| $\mathbf{1}$ | 5.6 | 65 | + | + |
| $\mathbf{2}$ | 5.7 | 65 | + | + |
| $\mathbf{3}$ | 5.75 | 64 | + |  |
| $\mathbf{4}$ | 5.3 | 40 | $+(\mathrm{down})$ | $+(\mathrm{down})$ |
| $\mathbf{5}$ | 5.4 | 39 | + | + |
| $\mathbf{6}$ | 5.5 | 40 |  | + |
| $\mathbf{7}$ | 6.0 | 42 | + |  |
| $\mathbf{8}$ | 6.0 | 42.5 | + | + |
| $\mathbf{9}$ | 5.5 | 33 | + | + |
| $\mathbf{1 0}$ | 5.6 | 32.5 | + | + |
| $\mathbf{1 1}$ | 6.1 | 33 | + |  |
| $\mathbf{1 2}$ | 5.8 | 25 | + |  |
| $\mathbf{1 3}$ | 6.8 | 24 | + |  |
| $\mathbf{1 4}$ | 6.9 | 23 |  | + |
| $\mathbf{1 5}$ | 6.9 | 17.5 | + | + |
| $\mathbf{1 6}$ | 6.9 |  | + | + |

(MALDI-TOF MS/MS). The identification of the various protein spots differentially regulated would be helpful in further functional characterization to HBx interference.

### 3.4.1 Mass Spectrometry Results

$200 \mu \mathrm{~g}$ of total proteins were loaded for a separate 2 DE analysis for both Genotype B and C HBx transfected Chang liver cells for the purpose of Mass Spectrometry. Spots were matched against those previously identified in Table 3.1 and selected spots were chosen for an initial MS analysis.

4 protein spots (namely spots $9,11,13$ and 14) were sent for Mass Spectrometry utilizing MALDI-TOF MS/MS (National University of Singapore, Protein and Proteomics Center) and the trypsinized peptide fingerprints (See Figure 3.11) were identified by match to database MASCOT. Spot 13 and 14 (fingerprints 1 and 2 respectively) found matches with human proteins namely Triosephosphate Isomerase and Ran, a GTP binding protein. Spots 9 and 11 (fingerprints 3 and 4 respectively) did not have distinct peaks and found no matches with Homo sapien sequences in database.

### 3.5 Discussion

In recent years, more emphasis have been placed on HBV genotypes in relation to their disease prognosis and treatment responses (Fung et. al., 2004, Kao et. al., 2000, 2002). In particular patients carrier of HBV genotype B seem to exhibit a less aggressive progression to HCC. As seen in our multialignment of database sequences (Refer to
 4700 Reflector Spec \#1 MC $\Rightarrow>B C \Rightarrow$ AdvBC $(32,0.5,0.1)=>N R(2.00)[B P=855.1,408]$


Appendix Figure 9.1), sequences were conserved within specific genotypes (B or C) with ~95 \% identity at nucleic acid level whereas differences between the two genotypes were greater at $\sim 9.4 \%$. These differences could potentially play a role in the differential HCC progression between genotypes.

Based on the multialignment, PCR primers were designed for genotyping (Mizokami et. al., 1999). Instead of described RFLP patterns by enzymatic digestion, nucleotide sequencing was carried out to identify restriction sites in silico. Our modified methodology allows accurate genotyping without the potential ambiguity in enzymatic digestion. In addition to sequencing HBsAg in genotyping, the relatively highlyconserved HBx sequences (as discussed later) of both genotypes were also compared to further confirm the genotype.

Interestingly, an increased tendency in a G1896A mutation in genotype B HBV sequences compared to C was observed (Appendix Figure 9.1). This G1896A mutant, located in the Pre-Core region has been previously associated with the early seroconversion of genotype B patients, hence resulting in a less severe and slower liver disease progression (Sumi et. al., 2003). The G1896A mutation occurs at $80 \%$ (4 out of 5) rate in genotype B patients compared to $20 \%$ (2 out of 8 ) in genotype C carriers. Further investigation on this mutation may provide new information on its role in viral replication and/or disease progression.

### 3.5.1 Cloning of Replicative Genome of Specific Genotypes

To date, the replicative HBV genome (rHBV) of genotypes B and C were successfully constructed (Figure 3.2 panel B). Sequencing within the viral genes coding for HBsAg, HBx and Core (Appendix Figure 9.1) was aligned with database. Sequencing results showed that it was indeed of the respective genotypes as computed in silico. Random nucleotide mutations, albeit low in occurrence, were also detected throughout the genome, possibly reflective of the intrinsic viral evolution in the particular serum sample. Such mutations could be common in chronic HBV carriers, as the viral genome frequently mutate either in response to drug treatment, host immune response, or naturally due to the lack of proof-reading of HBV polymerase. The impact of these mutations on disease progression remains to be established.

A 102bp in-frame deletion encompassing the C-terminal PreS1 and the N terminal Pre-S2, was observed in the constructed genotype B replicative genome (Appendix Figure 9.3). The cloning of this mutant genome in its replicative form should be useful for future studies on its effect in viral replication, as seen in other deletions in the PreS region (Chen et. al., 2002), Besides rentention of HBsAg L protein, the Cterminal of Pre-S1 also plays a role in the trans-inhibition of M and S protein secretion. Hence, such deletion may for example result in the reversal of inhibition in HBsAg biogenesis (Gallina et. al., 1995), potentially resulting in disease progression. The importance of PreS region in viral replication cycle has been further highlighted by its activity in transcription activation which might contribute to the pathogenesis of HCC (Kim et. al., 1997).

### 3.5.2 Cloning of HBx of Genotypes B and C

A more straightforward alternative in our project was to study the effects of HBx , based on its widely reported involvement in cellular signaling and HCC development. HBX cloned into pCDNA3.1+ from viral DNA of genotypes B and C were sequenced and translated in silico for comparison. As seen in Figure 3.7 (A), most of the amino acid of difference belongs to the same functional group. Noticeable differences can be observed in the serine / proline rich region approximately a.a. 31-50. It is likely that genotype B which has a higher amount of proline could have a different structural configuration compared to translated genotype C HBx (more serine residues in the same stretch). This difference may be translated in different functional role resulting in disease progression variations. Proline residues potentially have restricted rotational freedom and is non-polar compared to serine and this proline-rich motif is characteristic of SH 3 domain binding motif, PXXP (where X stands for any amino acids). (The differences observed are conserved in database sequence alignment as observed in Appendix Figure 9.4)

Beside the serine/proline rich region, a tri-peptide difference was seen at 86-89a.a. In genotype B, it is a His-Arg-Asn while Arg-Gln-Val in genotype C. Although these a.a. are essentially hydrophobic or hydrophilic in nature, the Histidine has an imidazole side chain which could potentially alter the structural and functional role of the region. Consistently, these two sets of differences were also observed in database sequences translated HBx comparison (Figure 3.7 panel A)

Because of their structural differences, these apparently genotype-specific differences may have functional impact on the role of HBx . This seems to be supported by our preliminary cell line studies and will be elucidated in later chapters.

### 3.5.3 Two-Dimensional Electrophoresis

Although significant risk factors for hepatocellular carcinoma (HCC) are well known from epidemiological studies, early diagnosis is difficult and HCC remains a leading cause of cancer death worldwide particularly in Asia (Bosch et. al., 1999). Generally, the development of HCC is associated with multiple changes at the mRNA and/or protein level, some of which have been used as prognostic markers (ZeindlEberhart et. al., 2004). One of such markers, the $\alpha$-fetoprotein (AFP), has been widely used in diagnosis and monitoring for HCC (Tan et. al., 2003). However, 70\% of HCC patients are found to have levels of AFP lower than the threshold concentration (500 $\mathrm{ng} / \mathrm{ml}$ ) defined for diagnosis of HCC (Seow et. al., 2001). The variability of AFP has also been suggested by its high concentration in chronic HBV carriers without HCC.

While the reliability of HCC diagnosis may be improved by a combination of AFP in conjunction with other newly-found bio-markers (e.g. melanoma-associated antigen gene) (Qin and Tang, 2004), its significance has been drastically decreased by the stage of HCC development which often would have been too advanced for effective diagnosis and treatment (Mou et. al., 2002). Recent development in techniques that allow for the parallel analysis of the expression of large number of genes, at either RNA or protein levels, has opened new opportunities in identifying novel markers for HCC
diagnosis. Some examples include gankyrin that has been identified through cDNA microarray and found to be highly expressed in hepatoma (Higashitsuji et. al., 2000). Likewise, the significant expression of 90 other genes identified through genome-wide expression profiling has been correlated with the metastasis of HCC (Cheung et. al., 2002; Neo et. al., 2004). Concurrently, several protein markers including aldose reductase-like protein and cytokeratin 19 have been identified through large scale proteomics analysis in either HCC-derived cell lines (Ding et. al., 2004, Seow et. al.,2000, Zeindl-Eberhart et. al., 2004), or clinical serum samples (He et. al., 2003, Steel et. al., 2003).

Despite these advances, two issues remain to be solved. First, the source of these recent investigations has been mainly the HCC-derived cells which are reflective of a late / end stage of HCC development. This implies that the identified markers may not be useful in the context of early diagnosis / prognosis. The second important issue concerns the identification of new markers from clinical samples. Due to variation intrinsic to the clinical samples involved in earlier studies, the general use of these markers in HCC diagnosis may also prove to be not as accurate. One possible improvement in the identification of HCC biomarkers useful for early diagnosis and effective treatment may be to incorporate external factors that are closely associated with the initial onset of HCC. These include noticeably HBV viral infections, which account for more than $80 \%$ of HCC cases in Asia (Chen and Oon, 1999, Wang et. al., 2002). In the case of HBV infection, the risk of developing HCC for chronic HBV carriers faced an increased by a factor of 100 , the risk in developing HCC when compared with a non-infected individual (El-Serag et. al., 2001).

HBV infection and replication results in chronic liver injury including inflammation, liver regeneration, liver fibrosis and cirrhosis which highly predisposes healthy liver cells to HCC. However, not all HBV-related HCCs are a result of liver cirrhosis which implies that HBV may have direct hepato-carcinogenic properties. Besides the chromosomal instability caused by HBV genome integration into host genome (Slagle et. al., 1991, Robinson W.S., 1994, Idilman et. al., 1998), the smallest HBx protein could also influence the onset of HCC by promoting the survival and growth of transformed Hepatocytes as shown in earlier studies (Andrisani and Barnabas, 1999, Birrer et. al., 2003; Chen et. al., 2000a).

In addition to the involvement of the elusive HBx in HCC, HBV genotypes have recently been linked with the clinical outcome of viral infection. For genotypes B and C that are predominant in Asia, clinical studies have suggested that genotype B is often associated with HCC in non-cirrhotic HBV carriers. On the other hand, genotype C patients were linked with more severe liver disease often including cirrhosis and eventually leads to development of HCC (Kao, 2002, 2003). The purpose of our study is to establish a proteomics approach to identify cellular proteins associated with exposure of HCC inducing agents, namely the HBV infection. In particular, our aim is to determine whether significant cellular proteins differences in expression could be identified when exposed to genotype specific HBx .

To mimic the early infection by HBV and characterize early cellular events to HBV infection in particular associated with HBx, Chang liver cells were transfected with pCDNA3.1+ carrying HBX of either genotype B or C. Such cellular events are being analyzed by 2DE from two different perspectives. First, the global analysis of cellular proteins in response to cellular exposure to HBx should provide valuable information on the role of HBx in cellular signaling, and its role in HCC development. On the other hand, similar proteomics analysis on cell culture medium should lead to the identification of secreted proteins in response to the cellular exposure to HBx . These proteins may have potential, upon validation in clinical samples, as early biomarkers of HCC diagnosis which remains a key challenge in the effective treatment of HCC.

We have recently established the HBV replication system in rat primary hepatocytes. Early investigation suggested that HBV replication occurs as efficiently as in HepG2 cells. In addition, apoptosis induced by HBV replication as observed in HepG2 cells was also detected in the primary hepatocytes (data not shown). This primary hepatocytes will provide crucial validation of the potential biomarkers identified in this project.

In our preliminary proteomics analysis, each protein extract from HBx transfected and control cells were ran three times. This should effectively reduce the number of falsepositive spots of proteins due to the high sensitivity of silver staining. At this stage, only the spots that are up- or down-regulated in all 3 gels have been included for further analysis. As seen in Table 1, most differences were detected from cells transfected with

HBx of genotype B - namely spots $3,7,12,13,14$ and 15 . Differences were also identified from cells transfected with HBx of genotype C , namely the spots $6,8,10$ and 16. Spots identified from our analysis were sent for MS for their matching with the MASCOT database and their eventual identification.

Similar analysis will also be carried out in cells transfected with the replicative HBV genomes of genotypes $B$ and $C$, once these constructs are ready. This should provide useful information on the role of HBx as opposed to the whole HBV genome in HCC development could be assessed.

### 3.5.4 Mass Spectrometry Results

Spots 9 and 11 (corresponding to Fingerprints 3 and 4, Figure 11) did not show any matches to Homo sapien proteins by Mass Spec analysis (MS). This could be due to poor sample preparation during the staining and techniques in preparing for MALDI-TOF. Alternatively, these may be potential new proteins. Their identification could be improved by better sample preparation or N -terminal sequencing.

Spot 13 (corresponding to Fingerprint 1), was closely matched to Homo sapien Triosephosphate isomerase (TPI). It is a common housekeeping enzyme which takes part in the inter-conversion of dihydroxyacetone phosphate and glyceraldehydes-3-phosphate, in the glycolysis pathway (Maquat et. al., 1985). However, it has been reported that an alteration of the concentrations of TPI could lead to disease states. For instance, it has also been shown to be upregulated in renal cell carcinoma (Lichtenfels et. al., 2003) and
hepatoma (Liang et. al., 2002, Takashima et. al., 2003). The appearance of the 2 spots, up and down (slight difference in molecular weight) could be due to the different isoforms of TPI (Maquat et. al., 1985). It is possible that cellular exposure to HBx of genotype B results in the malfunction of certain isoforms of TPI and thereby causing abnormal malignant transformation of hepatocytes. Consistently, cells transfected with the empty vector (pCDNA3.1+) or HBx of genotype C did not show any differences as seen in Figure $3.10(\mathrm{~F})$ spot 13.

Spot 14, corresponding to Fingerprint 2, was matched to Ran. Ran is a small GTP binding protein and part of the Ras protein superfamily (Vetter et. al., 1999). Ran plays a key role in the nuclear import of nuclear destined proteins. It remains to be determined whether HBX interacts directly with Ran, as it might posses RanBD (Ran binding domain sequences) (Hartman and Gőrlich, 1995). Such interaction may result in abnormal activation of Ran, causing an influx of normally cytoplasmic proteins into the nucleus thereby altering the transcriptional activities. Ran's morphological changes as seen in Figure 3.10 could possibly be attributed to the C-terminal alteration frequently associated with Ran, resulting in a conformational change. HBX could also interact with Ran at the C-terminal substituting for RCC1 which catalyzes the GTP bound to Ran to exchange for GDP resulting in the downstream transformation in hepatocytes.

Taken together, a complete sequence of proteomics approach has been successfully established in our study. Starting from the in vivo simulation of viral infection with genotype-specific HBV genes (HBx) or whole genomes (in a near future),
this approach covers the 2DE and MS analysis leading to the identification of cellular proteins associated with HBV infection. Further MS analysis on other genotype-specific spots should provide useful information on our understanding of cellular signaling events in response to $\mathrm{HBx} / \mathrm{HBV}$ infection.

### 3.6 Conclusion

We have established a cell-based proteomics approach for identifying early cellular proteins in response to transfection by mammalian expression vector carrying gene of interest, particularly HBx. This approach will be extended to cells transfected with replicative HBV genomes of specific genotypes. Our results revealed that specific cellular proteins were associated with exposure to a particular genotype of HBx. Analysis by MALDI-MS on different proteins spots would be helpful in the molecular identification and further functional characterization of these cellular proteins. One of the applications of our approach would be to search for new markers for early diagnosis of HCC. In this context, the recently developed primary hepatocyte system will be transfected with genotype specific HBV genome in their replicative form (Chen et. al., 2000b). The infected culture medium would then be analyzed by the proteomics approach reported in our study to identify secreted proteins associated with a particular genotype of HBV. Since these soluble proteins would be identified in an environment simulating the initial stage of HCC development (primary hepatocytes coupled with HBV replication), they may have a potential to be used as early diagnostic HCC markers. Such a potential could be validated by testing their presence in HCC cells from clinical samples. In particular, the correlation between any secreted proteins in primary hepatocytes that are
associated with specific HBV genotype, and their presence in HCC cells associated with the same genotype, should further strengthen the potential of these proteins in early HCC diagnosis.

For Chang liver cells transfected with HBx of genotype B or C, a number of cellular proteins were found to be associated with specific genotypes. Further validation should be carried out by repeating similar analysis in primary hepatocytes. MS Analysis led to the identification of two of such proteins, namely Triosephosphate Isomerase (TPI) and Ran (Ras-related small GTP binding protein). These proteins could well be part of the early cellular response to HBx . A complementary comparison with proteomics analysis from cells transfected with replicative HBV genome should not only lead to additional cellular proteins, but also shed new lights on the specificity of TPI and Ran in the context of viral infection.

Although the successful cloning of replicative HBV genome of genotype B contains a 102bp in-frame deletion in PreS (intrinsic of serum sample), this mutant genome could provide useful information on the impact of this deletion on viral replication. Viral fragments of wild type (without internal deletion) genotype B and C have now been cloned in TOPO vectors and their respective replicative genomes are being constructed. Proteomics analysis, similarly to that from cells transfected with HBx, will then be carried out. In addition to the identification of cellular proteins in response to HBV infection, such analysis should also provide useful information on the role of HBx in HCC development.

Another aspect in our future direction might be to identify secreted proteins in response to HBV infection or exposure to HBX. Such secreted markers could potentially be used in early detection of HCC in clinical context, as current diagnostic marker AFP generally leads to the detection of untreatable late stage HCC. To this end, a modified protocol has recently been established in the lab, allowing successful 2DE analysis of cell culture medium (Appendix Figure 9.5).

Taken together, our data indicated that the proteomics approach could be used to identify cellular proteins associated with genotype-specific HBx. The findings would shed new lights in our understanding of early events of HCC development associated with HBV infection.

## Chapter 4 <br> HBx Interaction with Src Homology-3 Domain Containing Proteins

HBx, a non-structural viral protein of HBV plays important role in various signaling pathways as well as viral replication. It is now known that HBx does not bind DNA directly but may interact with cellular proteins such as CREB, for activation of downstream signaling cascades. Mechanisms of interaction between HBx and cellular proteins are not thoroughly characterized probably due to the lack of functional motifs that have been identified. Here, we attempt to understand if the proline-rich motif within the HBx as described in Chapter 3 might have a role as a SH3-binding protein in its interaction with cellular proteins. We made use of mass screening using a SH3 domaincontaining membrane and a probe generated from HBx and a number of proteins were identified.

### 4.1 Proline-rich Region (SH3 Binding Motif) in HBx

### 4.1.1 Identification of a Proline-rich Region in HBx

HBx is the smallest viral protein encoded by HBV genome and comprises of 154 amino acids with a molecular weight of $\sim 17 \mathrm{kDa}$. As highlighted previously in Figure 3.5, HBx showed in particular, high variability in the region between amino acids 31 to 43 . This was surprising in that the rest of the HBx amino acid sequences were well conserved. The differences were however observed amongst all genotypes as seen in comparison between cloned genotype B and C HBx (Figure 3.7 panel A), showing the alignment of HBx database sequence compared to the HBx cloned from the HBV viral DNA extracted from clinical samples (Courtesy of SGH and NUH). The alignment further highlighted not only the potential importance of the conserved sequences among HBx proteins, but also the divergent region between a.a. 31 to 43 .

Many groups have been studying the effects of HBx on apoptosis and carcinoma and have yielded contradicting results (Shintani et. al., 1999, Hsieh et. al., 2003, Shirakata and Koike, 2003, Chen et. al., 2004). It is possible that the differences in their results are due to the different genotypes of HBx used in their experiments. The differences in amino acid sequence might be the key to the differing disease outcome via interacting with differing set of cellular proteins. Indeed, structural differences have been shown to be important for protein-protein interaction, thereby allowing downstream signaling transduction cascade activation or inhibition.

### 4.1.2 "PXXP" Motif

From the proline rich domain, we observed that genotype B HBx specifically contained a "PXXP" motif (Nguyen et. al., 1998). This "PXXP" motif has been specially found in SH3 domain interacting proteins. In addition, various other non-canonical "PXP" ( $\beta$ PIX) and "PXXXP" motifs were observed in the various genotypes of HBx. To elucidate the importance of these proline-rich hyper variable region in their downstream cellular function, we proceeded to screen for potential interacting SH3 domain-containing proteins with these proline-rich moiety. To this end, we made use of the commercially available TranSignal SH3 domain arrays (Panomics) (Figure 4.7), which have been previously used to show positive interaction of Tec with cellular proteins (Jiang et. al., 2004). The array consists of a nitrocellulose membrane dotted in duplicates with the expressed SH3 domains of various known SH3 domain-containing proteins. Positive interactors are to be detected by the use of the supplied anti-HIS antibodies. False positives are to be distinguished by the detection of only positive Western results on only
one of the two dots on the membrane while negative results showed no binding on the assay or spot on Western at all. Based on the principle of detection, the "PXXP" domain from HBx was expressed as a HIS tag containing protein, prior to the hybridization experiment with the SH 3 domains on the membrane.

### 4.1.3 Construction of $\mathbf{H B x}$ Proline-rich Probe

It is also important to note that various attempts by others as well as ours (unpublished data) have failed to express full length (FL) HBx by bacterial expression in the soluble form. Expressed HBx FL was always found in the pelleted insoluble fraction and some groups have attempted to refold these proteins but the efficiency remained low (Marczinovits et. al., 1997). A recent report by Marczinovits et. al. in 1997, showed that the N-terminal truncation improved expression of HBx by $20 \%$. Our attempt to express and purify the GST-fusion HBx FL construct was shown in Figure 4.1. From the Coomassie Blue stained SDS-PAGE, the expression of HBx FL fusion of GST (43 kDa) was detected only in the insoluble pellet fraction despite various conditions such as low temperature of induction ( $16^{\circ} \mathrm{C} 8 \mathrm{hr}$ ) or high OD (OD 1.0 at 600 nm ) prior to induction. The identity was confirmed by Western analysis using monoclonal anti-His antibodies (PENTA) to detect the incorporated C-terminal 6 X HIS. As the insoluble protein is not suitable for subsequent analysis, we generated a construct consisting of the sequences from HBx ranging from amino acid 19 to amino acid 57 (as highlighted in Figure 4.2 panel A). It has been shown previously in another construct of PAK1 (Zhao et. al., 2000) that a 18 a.a. GST-fusion construct of PAK1 retained their binding of the PAK1-
(A)

(B)


Figure 4.1:
Expression of GST-HBX FL-HIS construct. Constructs of Full length HBX in pGEX-5X-1 with C-terminal 6XHIS was transformed into BL21(DE3) and expressed in various conditions (illustrated below) (A) Coomassie Blue staining of the gel after Semi-Dry transfer showed that the expression of HBX (depicted by the red arrow) at the expected 42 kDa in lanes 2,4 and 6 . This corresponds to the insoluble fractions of the various expressed proteins. This is confirmed by (B) Western analysis using mouse anti-HIS antibodies (PENTA) followed by Goat antimouse conjugated with HRP.

Lanes 1, 3 and 5 are the soluble fractions while Lanes 2, 4 and 6 are the insoluble (pellet) fraction. Conditions for Lanes $1 \& 2\left(16^{\circ} \mathrm{C}\right.$ overnight 1 mM IPTG), $3 \& 4$ $\left(30^{\circ} \mathrm{C}, 6\right.$ hours, 1 mM IPTG) and $5 \& 6\left(37^{\circ} \mathrm{C}, 4\right.$ hours, 1 mM IPTG)
(A)

|  |  | $1>70$ |
| :---: | :---: | :---: |
| A HBX | (1) | MAARLYCQLDPSRDVLCLRPVGAESRGRPLAGPLGTLSSPSPSAVPADHGAHLSLRGLPVCAFSSAGPCA |
| B HBX | (1) | MAARLCCQLDPARDVLCLRPVGAESRGRPLPGPLGALPPASPPIVPTDHGAHLSLRGLPVCAFSSAGPCA |
| C HBX | (1) | MAARVCCQLDPARDVLCLRPVGAESRGRPVSGPFGTLPSPSSSAVPADHGAHLSLRGLPVCAFSSAGPCA |
|  |  | 71140 |
| A HBX | (71) | LRFTSARCMETTVNAHQILPKVLHKRTLGLPAMSTTDLEAYFKDCVF KDWEELGEEIRLKVFVLGGCRHK |
| B HBX | (71) | LRFTSARRMETTVNAHRNLPKVLHKRTLGLSAMSTTDLEAYFKDCVFNEWEELGEEIRLKVFVLGGCRHK |
| C HBX | (71) | LRFTSARRMETTVNARQVLPKVLHKRTLGLSAMSTTDLEAYFKDCVFTDWEELGEETRLKIFVLGGCRHK |
|  |  | $141 \quad 154$ |
| A HBX | (141) | LVCAPAPCNFFTSA |
| B HBX | (141) | LVCSPAPCNFFTSA |
| C HBX | (141) | LVCSPAPCNFFTSA |

(B)

| 19 aa | 57 aa |  |
| :---: | :---: | :---: |
| GST | HBx Proline <br> Rich Region | 6 X HIS |

Figure 4.2:
Alignment of HBx of genotypes A, B and C and design of probe for screening SH3 domain array
(A) Depicts the alignment of HBx of 3 genotypes of HBV , namely Genotypes A, B and C. As demarcated, the proline-rich highly variable region was flanked by corresponding amino acids and cloned into pGEX-5X-1 vector generating a GSTfusion protein as seen in the schematics. Yellow codes for similar residues, Blue codes for consensus residue at particular position and the black letterings codes for different residues at particular position.
(B). This probe was to be used for detection of interacting cellular proteins using the TranSignal SH3 protein Array (Panomics, USA). As required, 6X Histidine tag was added at the C-terminal for detection.
interacting protein, Nck1 through the SH3 binding motif. Hence, we constructed a HBx partial sequence as a fusion of GST using pGEX-5X-1 and incorporated a C-terminal 6 X HIS for subsequent detection (The probe design schematics was depicted in Figure 4.2 panel B). The constructs were entirely sequenced to ensure that the HBx partial fragment was cloned in frame with the GST. The expression of this GST-fusion protein was optimized at $37{ }^{\circ} \mathrm{C} 1 \mathrm{mM}$ IPTG as shown in Figure 4.3 (A). Furthermore, proper expression was evidenced by the detection of the 6 X HIS designed at the C-terminal end of the fusion construct by Western analysis (Figure 4.3 panel B) using mouse monoclonal anti-HIS antibodies (PENTA). Similar constructs for genotypes B and C were properly expressed and detected as seen in Figure 4.4. In order to study the importance of the proline residues within HBX, we made use of QuikChange site-directed mutagenesis (Stratagene) to mutate the proline residues to alanine. These mutations remove the amino acid makeup of the original amino acid sequence and hence potentially abolished its binding to SH3 domain. The mutagenesis was depicted in Figure 4.5 where the prolines underlined by asterix were replaced with alanine using specifically designed oligonucleotides. In order to obtain pure interacting proteins, GST microspin purification columns, utilizing the Sepharose 4B beads (GE Healthcare) were applied for the purification of the GST-fusion proteins. As observed in Figure 4.6, relatively pure fusion protein was generated after purification and was henceforth used for affinity capture purification of GST-PXXP-HIS as well as GST-AXXA-HIS proteins for future analysis. The expressed protein used for probe is the 31 kDa fusion of GST.
(A)

(B)


Figure 4.3:
Bacterial expression of GST-PXXP-HIS. As evidenced by (A), bacterial expression of the GST-PXXP-HIS construct (lane 2) were correct as seen by the increase in size from lane 1 which depicts GST alone expression from pGEX-5X-1 vector. Sequencing confirmed the entire length was in frame with the GST moiety. Also, the in-frame expression of the construct was evidenced by the use of mouse monoclonal anti-His antibodies (PENTA) shown in (B). Only the correct expression of the sequences from the GST-PXXP-HIS will result in proper expression of the 6 X His residues included in the $\mathbf{C}$-terminal end.
(A)

(B)

(C)


Figure 4.4:
Expression of GST-PXXP-HIS constructs of HBx genotypes B and C. Lanes 1, 2 and 3 depicts the un-induced total protein lysates of BL21 (DE3) of GST, GST-PXXPHIS (Genotype B) and GST-PXXP-HIS (Genotype C) respectively. Concurrently, lanes 4,5 and 6 depict the induced ( $37{ }^{\circ} \mathrm{C}, 4 \mathrm{hr}, 1 \mathrm{mM}$ IPTG) total protein lysates from the 3 described above respectively. (A) Coomassie blue staining of $\mathbf{1 2 \%}$ SDSPAGE after semi-dry transfer. (B) and (C) depicts Western analysis using Mouse anti-HIS (PENTA), to detect for the proper expression of genotype $B$ and $C$, and Goat anti-GST (Santa Cruz Biotech) to detect for all constructs, including GST alone.
rHBV 28 a.a.
57 a.a. Genotype A $\underset{*}{\operatorname{RPLAGPLGTLSSPSPSAVPADHGAHLSLRGL}} \underset{*}{*} \underset{*}{\operatorname{*in}}$


Genotype $\underset{*}{\text { RPVSGPFGT }} \underset{*}{\operatorname{LP}} \underset{*}{\operatorname{SPS} S S S A V P A D H G A H L S L R G L}$

## Figure 4.5:

The above shows the mutation strategy to remove any proline rich motifs present in the various type of GST-PXXP-HIS cloned. The area with a red $*$ indicates the area of mutation. Stratagene site-directed mutagenesis kit was made use of for the mutagenesis of these clones. These clones will be termed GST-AXXA-HIS and serve as the negative control for the proline rich constructs.

## Color Coding

(1) Yellow : Completely conserved residue at given position.
(2) Blue : Consensus residue derived from a block of similar residues at a given position.
(3) Green : Residue weakly similar to consensus residue at given position.
(4) Black letterings : Non-similar residues.
(5) Green letterings: Residues weakly similar to consensus residue at the particular position.


Figure 4.6:
Purification of expressed GST-PXXP-HIS, using GST microspin purification module (Sepharose 4B beads) (GE Healthcare). Lane 1 shows the crude lysates from the IPTG induced B121 cells expressing GST-PXXP-His (~31 kDa). Lane 2 depicts the flow through after GST-fusion proteins have bound to the beads. Lane 3 shows a pooled sample from the 3 washes with $1 \times$ PBS. Lane 4 shows the purified GSTfusion protein of 31 kDa after elution. Purified GST-fusion proteins were used for interaction analysis.

### 4.2 TranSignal SH3 Domain Array (Panomics)

### 4.2.1 Identifying Interaction with HBx PXXP Motif

For this analysis, we made use of TranSignal SH3 domain array II, with various immobilized SH3 domain-containing proteins as listed in Figure 4.7, (A) the schematics of the SH3 domain used was depicted (B) Full names of the list of proteins SH3 domains on the membrane were listed. The principle of hybridization was similar to Western blot analysis, relying on the affinity of the proline-rich probe for the membrane-immobilized SH3 domains instead of the antigen-antibody recognition. As depicted in Figure 4.8 (A) and (B), many proteins were found to interact with the GST-PXXP-HIS probe. The results was tabulated in Table 4.1. Proline-rich regions have been shown to interact with SH3 domains promiscuously, but specificities of the motif have also been reported (Alexandropoulos et. al., 1995). In some cases, residues surrounding the PXXP SH3 binding motif e.g. Arginine may also be involved in SH3 / PXXP interaction. (Kay et. al., 2000). Our data provided the first evidence of the proline-rich domain within HBx which served as a SH3-binding motif. The significance of this proline-rich region as a SH3 binding motif was further supported by the drastic reduction of interaction, when the mutated probe GST-AXXA-HIS was used. This highly variable domain of the otherwise conserved HBx of only 154 a.a., might play important roles in the differential disease outcome of various genotypes of HBV infection through their interaction with SH3 containing proteins. More importantly, many SH3 domain containing proteins play important role in normal cellular function such as maintenance of cell morphology (Koh et. al., 2001) as well as cellular signaling cascade (Pawson and Scott, 1997, Tan et. al., 1999). As seen in Fig 4.8 (A) and (B), c-Src interacted strongly with both genotypes B
(A)

|  | 12 | 34 | 5 6 | 78 | $9 \quad 10$ | 1112 | 1314 | 1516 | 1718 | 1920 | 21 | 22 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| A | Abl2B | GRB2L-D1 | ABL2 | CCBA | CRKL-D1 | CSKP | NCK1-D2 | NE-DLG | NOF2-D1 | OSF | po |  |
| B | P13 $\alpha$ | SP93 | STAC | Tec | TRIP10 | PIG2 | ARH6 | BCA1 | BIN1 | EFS | po |  |
| C | GRAP-D1 | JIP1 | M3KA | MY7A | NCK2-D2 | NCK2-D3 | RHG4 | SH31 | SNX9 | UAS3 | pos |  |
| D | VAV2-D1 | VAV3-D1 | VAV3-D2 | VINE-D1 | VINE-D3 | c-Src | GST |  |  |  | po |  |
| E | pos | pos | pos | pos | pos | pos | pos | pos | pos | pos | po |  |

Schematic diagram of the TranSignal ${ }^{\text {m }}$ SH3 Domain Array II. The proteins on the array are spotted in duplicate. Histidine-tagged ligand has been spotted along the bottom (row E) and in duplicate along the right side of the membrane (column 21, 22). These spots are intended for alignment. Note that the notch is at the top, right-hand corner:
(B)

## SH3 domain list • TranSignal ${ }^{\text {TM }}$ SH3 Domain Array II

| POSTIION | DCMAIN | FULL NAME |
| :---: | :---: | :---: |
| A1, 2 | Abl2B | Abl interactor protein 2 |
| B1, 2 | PY3a | Phosphatidylinositol 3-kinase regulatory alpha subunit |
| C1,2 | GRAP-D1 | CRE2-related adaptor protein, SH3 Domain 11 |
| D1,2 | VAV2-D1 | Vav-2 protein,SH3 Domain ${ }^{\text {n }}$ (1 |
| A3, 4 | GRB2L-D1 | Qeb2-related adaptor protein 2, SH3 Domain ${ }^{\text {2 }}$ |
| B3, 4 | SP93 | Chamel associated protein of synapse-110 |
| C3,4 | IP1 | Cjun-amino-terminal linase interacting protein 1 |
| D3,4 | VAV3. D1 | Vav-3 protein, SH3 Domain ${ }^{\text {n }}$ |
| A5,6 | Abl2* | Abelson-related peoteins Arg |
| BS, 6 | SINC | Sexpeotein |
| C5, 6 | M3KA | Mitogen-activated protein kinase kinase kinase 10 |
| D5,6 | VAV3-D2 | Vav-3 protein,SH3 Domain 52 |
| A7, 8 | COA | Dhydropyridinesensiaive L-type, calcium channel beta-1 subunt: |
| B7, 8 | Tec | Tyrosire-protein linase Tec |
| C7, 8 | M 7 \%A | Myocin VIla |
| D7,8 | VINE-D1 | Vinexin,SH3 Domain $\# 1$ |
| A9, 10 | CRKL-D1 | CRK-like protein,SH3Domain E1 $^{\text {a }}$ |
| B9, 10 | TRIP10 | Cde42-interacting protein 4 |
| C9, 10 | NCK2-D2 | Cytoplasmic protein NCK2, SH3 Domain 22 |
| D9, 10 | VINE-D3 | Vinexin, SH3 Domain 53 |
| A11, 12 | CSNP | Feripheral plasma membrane protein CASK |
| B11, 12 | PXC2 | 1-phosphatidylinosilol-4,5-kesphosphatephosphodiesterase gamma2 |
| C11, 12 | NCK2-D3 | Cytoplasmic protein NCK2, SH3 Domain 23 |
| D11, 12 | c.Sice* | Cellular Rous Sarcoma viral oncogere homolog |
| A13, 14 | NCK1-D2 | Cytoplasmic protein NCK1,SH3 Domain 22 |
| B13, 14 | ARH6 | Eho guanine nucleotide exchange factor 6 |
| C13, 14 | RHCA | Eho-GTPase-activating protein 4 |
| D13, 14 | control | negative control |
| A15, 16 | NE-DLG | Presymaptic proteinSAP102 |
| B15, 16 | BCA1 | CRK-ssociatedsubztrate |
| C15, 16 | SH31 | SH3-cootaining GRB2-like peoteia 1 |
| D15, 16 | -* |  |
| A17, 18 | NOF2-D2 | Neutrophil cytosol factor 2, SH3 Domain \#2 |
| B17, 18 | BIN1 | Mycbox dependent interacting protein 1 |
| C17, 18 | SNX9 | Sorting nexin 9 |
| D17, 18 | *- |  |
| A19, 20 | CsF | Osteodast stimulating factor 1 |
| B19, 20 | EFS | Embeyoral Fym-associated substrate |
| C19, 20 | UAS3 | UBASH3A |
| D19, 20 | *- |  |
| This SH3 domainis akospotted onthe TranSigrapm SH3 Donain Array L |  |  |
| *This SH3 | ospotted on | nal ${ }^{\text {P4 SH3 }}$ DomainArrays I \& III. |

Figure 4.7:
TranSignal SH3 Domain Array II. (A) Schematics to show the proteins contained. Essentially, SH3 domains of various proteins identified to date were expressed by Panomics and spotted in duplicates on a nitrocellulose membrane (e.g. c-Src was spotted at position D 11 and 12). GST serve as the negative control for non-specific interaction at position D 13 and 14. To serve as positive controls, peptides containing Histidine which would be hence recognized by the anti-HIS antibodies was spotted at the right hand periphery (A 21 and 22 to E 21 and 22) as well as the base of the membrane ( $E 1$ to 20). These serve as good orientation points of the membrane. (B) shows a list of the protein.

(B)

(D)

Figure 4.8:
TranSignal SH3 domain Array II probed by GST-PXXP-HIS and GST-AXXA-HIS of HBx genotypes B and C. (A) and (B) represents the use of probe GST-PXXP-HIS (Genotype B) and GST-AXXA-HIS (Genotype B) on Array II respectively. (C) and (D) represents the use of probe GST-PXXP-HIS (Genotype B) and GST-AXXA-HIS (Genotype C) on Array II respectively. Red color boxes demarcates the proteins interacted by both the Genotype of GST-PXXP-HIS while blue boxes demarcates the negative control of this experiment consisting of the GST only. Black boxes demarcate a distinct difference in binding partner amongst others between the genotypes.

Table 4.1:
Interacting proteins identified through TranSignal SH3 domain array II. Probe used were GST-PXXP-HIS for genotypes B and C as well as their respective GST-AXXA-HIS mutants as established in Figure 4.5. As seen above, the columns and their respective SH3 domain coded for were identified from those that were nonreactive. Those that are found in the genotype are ticked on the right column(s). As observed, albeit the differences in amino acid sequences, most of the interacting proteins identified were similar except a few. Also, GST serving as an internal negative control did not show interaction.

| Column <br> $(\#, \#)$ | Domai <br> $\mathbf{n}$ | Full name | Genotype B | Genotype C |
| :--- | :--- | :--- | :---: | :---: |
| A (5, 6) | Ab12 | Abelson-related protein, Arg | $\sqrt{ }$ | - |
| A (19, 20) | OSF | Osteoclast stimulating factor 1 | $\sqrt{ }$ | $\sqrt{ }$ |
| B (7, 8) | Tec | Tyrosine-protein kinase, Tec | $\sqrt{ }$ | $\sqrt{ }$ |
| B (11,12) | PIG2 | 1-phosphatidylinositol-4,5- <br> biphosphate phosphodiesterase <br> gamma 2 | $\sqrt{ }$ | $\sqrt{ }$ |
| B (13,14) | ARH6 | Rho guanine nucleotide exchange <br> factor 6 | $\sqrt{ }$ |  |
| B (19, 20) | EFS | Embryonal Fyn-associated <br> substrate | $\sqrt{ }$ | $\sqrt{ }$ |
| C (13,14) | RHG4 | Rho-GTPase-activating protein 4 | $\sqrt{ }$ | $\sqrt{ }$ |
| D (7, 8) | VINE- <br> D1 | Vinexin, SH3 domain \# 1 | $\sqrt{ }$ |  |
| D (9, 10) | VINE- <br> D3 | Vinexin, SH3 domain \# 3 | $\sqrt{ }$ |  |
| D (11, 12) | c-Src | Cellular Rous Sarcoma viral <br> oncogene homolog | $V$ | $\sqrt{ }$ |
| D (13,14) | GST | Negative control | $\sqrt{ }$ | $\sqrt{ }$ |

and C of HBx and this interaction was dramatically reduced with mutation of the proline residues of the constructs to relatively inert alanine. This is another area which might be interesting as it has been previously postulated, that the HBx might potentially interact with c-Src resulting in downstream signaling cascade activating Src kinase (Klein \& Schneider et. al., 1997).

Interestingly, such proline-rich motifs have been previously found in Nef protein of Human immunodeficiency virus (HIV) (Hiipakka et. al., 2001) as well as NS5A protein of the Hepatitis C virus (HCV) (Tan et. al., 1999). Both of these proteins play similar role to HBx in that they are non-structural proteins (Greenway et. al., 1994, Reyes 2002) but yet play critical role in affecting both infectivity as well as disease outcome of their respective viral infection. Therefore, we hypothesize that there may be a unifying mechanism of viral infection making use of PXXP motif to interact with cellular SH3 domain containing proteins resulting in downstream disease outcome. This prompted us to pursue the potential importance of HBx highly variable region of proline-rich motif in disease causative effects. This might have great impact on disease outcome due to evoking differential cellular malfunctioning.

### 4.2.2 Cloning and Further Characterization of Interacting Proteins

From literature search, various proteins that interacts with GST-PXXP-HIS constructs were chosen by virtue of their implication in liver diseases, such as hepatocellular carcinoma and liver cirrhosis. It was also noted that only proteins that were expressed by the liver cells were chosen. The proteins that were chosen for studies were

Vinexin (Kioka et. al., 1999, Akamatsu et. al., 1999, Takahashi et. al., 2005) and Cortactin (Chuma et. al., 2004) which are important proteins, playing critical role in the maintenance of the cell cytoskeleton. These proteins were selected based on their importance in maintaining cellular morphology and function, and potential scale of impact on HBx binding. Reverse Transcription-Polymerase Chain Reaction was performed (using specifically designed oligonucleotides primers) on total RNA samples from HepG2 cells. For protein without commercial antibodies for the endogenous protein (e.g. Vinexin $\beta$ ), their coding region were cloned into mammalian expression vector, pXJ40HA, a kind gift from Dr Koh Cheng-Gee (NTU, School of Biological Sciences). For pulldown assays, pXJ40HA constructs were transfected into HEK293T cells and expressed proteins were collected as described in Section 2.5.3. The incorporated HA-tag served to facilitate detection using Santa Cruz Biotechnology monoclonal mouse anti-HA antibodies (Santa Cruz Biotech). Constructs were also cloned in pGEX-5X-1 for bacterial expression.

### 4.3 HBV and Cytoskeleton Changes

It has been shown that HBV replication directly alters the expression of key cytoskeleton-associated proteins which play key roles in mechanochemical signal transduction. Nevertheless, little is known on the correlation between HBV replication and the subsequent adhesion mechanism of HBV-replicating cells. During its replication, HBV makes use of components of host cell to synthesize various viral proteins as mentioned above. For example, the phosphoprotein HBcAg binds to the C -terminal region of actin-binding protein, which may modulate the function of membrane-bound
receptor and interfere with the mechanochemical signaling of adherent hepatocyte (Huang et. al., 2000). Moreover, HBx has been shown to enhance the transcription, translation and secretion of matrix metalloproteinase-9 and metalloproteinase-3 (Juang et. al., 2000, Chung et. al., 2002) which affects cell adhesion and migration of several types of cells (Yu et. al., 2005). Recent evidence also suggests the role of HBx in cellular apoptosis, a process involving major cytoskeletal disruption (Lara-Pezzi et. al., 2002).

### 4.3.1 Vinexin $\beta$ and HBx

It is important to note that Vinexin $\beta$ consist of the same SH3 domain, short of a N-terminal domain Sorbin Homology domain found in Vinexin $\alpha$ (Full length Vinexin) (Kioka et. al., 1999) (Refer to Appendix Figure 9.6). It should also be noted that HBx of genotypes B and C both interacted with Vinexin Domain III and only that of genotype C interacted with both domains on Vinexin (Tabulated in Table 4.1). Initial attempts to isolate Vinexin $\alpha$ from HepG2 cells by RT-PCR were not successful. Literature search showed that indeed, Vinexin $\beta$ was the only isoform expressed in liver \{Kioka et. al., 1999) whereas Vinexin $\alpha$ was found in most other tissues. Vinexin $\beta$ was amplified successfully from our HepG2 extracted total RNA, and cloned in pXJ40HA as well as pGEX-5X-1 (incorporation of C-terminal HA tag through oligo design). Cloned constructs were sequenced to ensure that the cDNA was cloned in frame and no mutations were generated in the PCR. As shown in Figure 4.9, Vinexin $\beta$ was expressed properly as evidenced by the Western blot analysis. The MW $\sim 62.6 \mathrm{kDa}$ (GST-fusion protein) was evidenced by Western blot using specific monoclonal anti-HA antibodies (Santa Cruz Biotechnology).

## (A)


(B)


Figure 4.9:
Expression of GST-VinexinßHA. GST-Vinexin $\beta$ HA was transformed into BL21(DE3) and expressed. $40 \mu \mathrm{~g}$ of total protein lysates from un-induced and IPTG induced BL21 (DE3) were ran on a 12 \% SDS-PAGE as depicted by Coomassie blue staining (A). Lane 1 depicts the un-induced GST-VinexinßHA. Lanes 2 and 3 depicts the expressed GST-Vinexin $\beta$ HA using 1 mM IPTG at two different induction temperatures at $30{ }^{\circ} \mathrm{C}(6 \mathrm{hr})$ and $37{ }^{\circ} \mathrm{C}(4 \mathrm{hr})$ respectively. As observed, there was basal expression of the VinexinßHA in un-induced (Lane 1) and no difference between expression level of the 2 varying temperatures of induction (Lanes 2 and 3). Western was carried out using Mouse anti-HA (Santa Cruz) followed by Goat antimouse, conjugated to HRP (Pierce). As correctly predicted, the fusion protein of ~ 62.6 kDa was observed in the induced forms Lane 2 and 3 as shown in (B) indicated by the red arrow.

### 4.3.2 Optimization of Vinexin $\beta$ Expression

Various attempts to optimize GST-Vinexin $\beta$-HA did not yield much improvement in increasing the expression efficiency. As observed in Figure 4.10, various temperatures of induction from $16^{\circ} \mathrm{C}$ ranging to $37^{\circ} \mathrm{C}$, as well as varying start of induction at OD 0.6 or OD 1 ( 600 nm ) did not improve nor decrease the expression efficiency. IPTG concentrations ranging from final concentration of induction from 0.1 mM to 1 mM also didn't show differences in expression efficiency. Finally, we decided to make use of the 1 mM IPTG $37{ }^{\circ} \mathrm{C}$ and 4 hours as basis of inducing Vinexin $\beta$ since $30^{\circ} \mathrm{C}$ induced sample showed equivalent concentration as evidenced by Western analysis in Figure 4.9. pXJ40HA cloned Vinexin $\beta$ was transiently transfected into HEK293T cells for overexpression by Effectene Transfection Reagent (QIAGEN). As depicted in Figure 4.11, mammalian expressed Vinexin $\beta$ showed a molecular weight of 36.6 kDa as evidenced by the Western analysis. GST-Vinexin $\beta$ was ran alongside for comparison.

### 4.3.3 Gel Overlay Assay for Vinexin $\beta$

As we have shown that HBx FL was essentially insoluble, a gel overlay assay was developed for confirmation of the determined protein-protein interaction. As depicted in Figure 4.12, GST, GST-PXXP-HIS and GST-AXXA-HIS of HBx genotype B were separated on a $12 \%$ SDS-PAGE and transferred to Hybond nitrocellulose membrane (GE Healthcare). $100 \mu \mathrm{~g}$ of GST-fusion protein of Vinexin $\beta$ was diluted in 1 X PBS and incubated for 4 hours at $4{ }^{\circ} \mathrm{C}$. Western analysis was then carried out to detect the HA tag found on the Vinexin $\beta$ probe. As


Figure 4.10:
Expression optimization of GST-VinexinßHA. The above shows the Coomassie Blue staining of various expression conditions of BL21 (DE3) Lanes 1,3 and 5 depicts the insoluble (pellet) proteins from expression. Lanes 2, 4 and 6 depicts the soluble fraction (supernatant) obtained from various temperatures at 1 mM IPTG induction, respectively at $16{ }^{\circ} \mathrm{C}(16 \mathrm{hr}), 30^{\circ} \mathrm{C}$ ( 6 hr ) and $37{ }^{\circ} \mathrm{C}$ ( 4 hr ) respectively. $40 \mu \mathrm{~g}$ of protein was loaded for the supernatant and a representative amount of insoluble pellet was loaded. Expressed GST-fusion protein at 62.6 kDa was observed and demarcated by the red arrow. Not much differences in expression was observed by induction at $30^{\circ} \mathrm{C}$ and $37^{\circ} \mathrm{C}$.


Figure 4.11:
Mammalian and bacterial expression of Vinexin $\beta$. Vinexin $\beta$ was expressed by cloning into $\mathrm{pXJ40HA}$ as well as cloning into pGEX-5X-1 with a C-terminal HA tag. pXJ40HA-Vinexin $\beta$ was expressed by transfection of HEK293T cells and lysis of cells for protein extraction 16 hours post transfection (lane 2). pGEX-5X-1 VinexinßHA was expressed and ran above in lane 3. Lane 1 depicts the total protein lysates from pXJ40HA empty vector transfected HEK293T cells. As above, both expression shows the correct molecular weight at $\sim 36.6 \mathrm{kDa}$ (Lane 2) and $\sim 62.6 \mathrm{kDa}$ (Lane 3) (GST-fusion) respectively.
observed in Figure 4.12 (B), a positive Western band was observed at 31 kDa in the lane for GST-PXXP-HIS. Contrasting this, the lanes for GST and GST-AXXA-HIS, did not show any presence of Vinexin $\beta$. This showed that that the interaction was specific and not due to interference from GST. We proceeded to ascertain if Vinexin $\beta$ expressed in mammalian cells can interact with the constructs. In this case, the HBx FL insoluble GST-fusion protein was used for interaction purpose. As observed in Figure 4.13 (B), mammalian expressed Vinexin $\beta$ showed similar profile for interaction with GST, GST-PXXP-HIS and GST-AXXA-HIS constructs as seen previously in Figure 4.12 (B). However, it was interesting to note that HBx FL insoluble protein also showed interaction with strong binding as seen in Figure 4.13 (B). Therefore, we have shown that HBx might interact with Vinexin $\beta$, through a proline-rich PXXP sequence and the SH3 domain. This interaction was abolished by generation of a proline-alanine HBx mutant, demonstrating the specificity of this interaction.

Vinexin $\beta$ has been shown to enhance cell spreading on fibronectin substrates. Our newly discovered HBx interaction with Vinexin $\beta$ suggested that this interaction may attenuate the original binding of Vinexin $\beta$ to cytoskeleton proteins and focal adhesion complexes responsible for cell adhesion kinetics. Hence, to determine the impact of such an interaction on HBV-host interaction in general, we decided to transfect HBV and see if it results in any differences in cellular adhesion. These changes were monitored by biophysical measurements.
(A)

(B)


Figure 4.12:
Bacterial expressed GST-VinexinßHA in Gel overlay assay. (A) An SDS-PAGE was ran which consists of $40 \mu \mathrm{~g}$ of lanes 1 (GST), M (marker), 2 (GST-PXXP-HIS) and 3 (GST-AXXA-HIS). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and blocked with $5 \%$ non-fat milk/ 1 X PBS overnight at $4^{\circ} \mathrm{C}$. The next day, $100 \mu \mathrm{~g}$ of expressed GST-Vinexin $\beta$-HA was diluted in 1 X PBS and subjected to rotation for 4 hr at $4^{\circ} \mathrm{C}$. After interaction, membrane was washed $2 X$ in PBS ( 10 min each) followed by detection using Western analysis by monoclonal anti-HA antibodies (Santa Cruz Biotech). Western result is depicted in (B). As observed, specific interaction was observed with a band at 31 kDa (indicated by red arrow), indicative of expressed GST-PXXP-HIS (lane 2). No band was observed at lane 1 (GST) or lane 3 (GST-AXXA). This shows that there was no interaction and that Non-specific interactions could be observed above 45 kDa and 66 kDa and might be indicative of other interacting proteins with Vinexin $\beta$.
(A)

(B)


Figure 4.13:
Mammalian expressed Vinexin $\beta$ in Gel overlay assay. (A) An SDS-PAGE was ran which consists of $40 \mu \mathrm{~g}$ of lanes 1 (GST), M (marker), 2 (GST-PXXP-HIS) and 3 (GST-HBX FL-HIS), 4 (GST-AXXA-HIS). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane and blocked overnight in $5 \%$ non-fat milk / 1 X PBS. Next, $100 \mu \mathrm{~g}$ of protein lysates from pXJ 40 HA -Vinexin $\beta$ transfected HEK293T cells, was diluted in PBS and subjected to rotation for 4 hr at $4^{\circ} \mathrm{C}$. After interaction, membrane was washed twice in $1 \times$ PBS ( 10 min each) followed by detection using Western analysis by monoclonal anti-HA antibodies (Santa Cruz Biotech). Western result is depicted in (B). As observed, specific interaction was observed with a band at 31 kDa and 43 kDa (indicated by red arrows), indicative of expressed GST-PXXP-HIS (lane 2) and GST-HBX FL-HIS (lane 3). No band was observed at lane 1 (GST) or lane 4 (GST-AXXA). This shows that there was no interaction. Non-specific interactions could be observed above 45 kDa and 66 kDa and might be indicative of other interacting proteins with Vinexin $\beta$.

### 4.4 Cell Adhesion Delay in HBV Replicating Cells

The communication between cell and extracellular matrix (ECM) is mainly mediated by integrin receptor which binds to Extracellular matrix (ECM) molecules with its extracellular domain and interacts with cytoskeletal proteins through its intracellular domain. HBV infection has been shown known to disrupt the integrin-mediated signaling cascades (Yeh et. al., 2000). For instance, HBx blocks the molecular linkage between the actin filament and cadherin complex and weakens the intercellular adhesion in a Src-dependent manner (Lara-Pezzi et. al., 2001a). Moreover, HBx reduces the expression of $\alpha_{1}$ and $\alpha_{5}$ subunits of integrin, impairs cell adhesion on fibronectin coated substrate and promotes cell migration (Lara-Pezzi et. al., 2001b). Therefore it is generally expected that the interaction between HBX and actin binding protein contributes to the morphological changes of hepatocytes through the alteration of the cytoskeleton organization. On the other hand, little is known on the effect of HBV replication on the biophysical mechanisms of cell adhesion on ECM.

The intricate interplay between cell adhesion kinetics, HBV replication and cytoskeletal protein modulation needs to be elucidated, especially with real-time biophysical measurements. Three cell types have been employed for our investigation including: 1. HepG2 cells transfected with a replicative HBV genome cloned in pcDNA3.1+ (rHBV) and co-transfected with pEGFP vector (Invitrogen, USA); 2. HepG2 cells with empty vector (pcDNA3.1+) and pEGFP vector; 3. normal HepG2 cells (control experiment) with pEGFP vector. The replicative HBV genome was constructed by
cloning a linear 1.1 X HBV viral genome into mammalian expression vector pcDNA3.1+ as described earlier. The linear genome contains the viral promoter at its 5' end and the region for termination of transcription at its 3' end as previously described (Chen et. al., 2000).

### 4.4.1 Cell Adhesion is Delayed in HBV Replicating Cells

Transfection was carried out using Effectene Transfection Reagent (QIAGEN) as per manufacturer's instructions. Briefly, $6 \times 10^{5} \mathrm{HepG} 2$ cells were seeded and cultured on a 60 mm dish (NUNC Inc., USA) under $37{ }^{\circ} \mathrm{C}$ and $5 \% \mathrm{CO}_{2}$ for twenty four hours before transfection. After adherent HepG2 cells reach 70 \% confluency, the cells were transfected with $2 \mu \mathrm{~g}$ of pcDNA3.1+ plasmid with or without replicative HBV genome (rHBV). In brief, the plasmid constructs are mixed with $16 \mu$ l of enhancer followed by 60 $\mu l$ of Effectene transfection reagent. After two hours of transfection, the medium was removed and rinsed twice with 1 X PBS, before addition of fresh medium. Transfected cells were maintained at $37{ }^{\circ} \mathrm{C}$ and $5 \% \mathrm{CO}_{2}$ for 48 hr to allow HBV replication. When transfected into HepG2 cells, HBsAg particles was produced in the cell culture medium two days post-transfection. These particles were specifically detected using IMX semiquantitative measurement (Abbott Laboratories). Transfected cells are detached by trypsinization as described above, pelleted and resuspended in fresh medium and immediately used for our biophysical studies.

Collagen is a common ECM protein which influences cell morphology, survival and proliferation (Wilkinson et. al., 2002). Cell attachment and spreading are mediated by the interaction between the adhesion receptors on cell surface and biological ligands on ECM (Shibutani et. al., 2000). For instance, different variants of integrin mediate the spreading of most anchorage-dependant cells on ECM coated substrates (Koivisto et. al., 1997, Feng et. al., 2005).

Cell adhesion dynamics is correlated with receptor-mediated signal transduction cascades such as the receptor expressions, ligand affinity, focal adhesion formation and cytoskeleton remodeling, etc (Palecek et. al., 1997, Gauet et. al., 2003). In this study, adhesion contact formation for HepG2 cells in response to HBV replication was measured. Figure 4.14 showed a series of C-RICM images of a typical normal HepG2 cell (A), HepG2 cell transfected with the empty pcDNA3.1+ vector (B) and HepG2 cell transfected with rHBV (C) against time of cell seeding on collagen coated substrate. For transfection with empty vector and rHBV, pEGFP plasmid was co-transfected into the cells. The result showed that normal cells and empty vector (pcDNA3.1+) transfected cells developed notable adhesion contact at 10 min of cell seeding (cluster-like structures on Figure 4.14 panels A and B) while similar adhesion contact formation was seen in cells transfected with rHBV 20 min after cell seeding (Figure 4.14 panel C). At 20 minutes after cell seeding, the adhesion contact area of normal cell and cell transfected by rHBV reached $173 \mu \mathrm{~m}^{2}$ ( $50 \%$ of the steady-state value) and $48 \mu \mathrm{~m}^{2}$ ( $9 \%$ of the steadystate value), respectively. The result supported that the extent of adhesion contact formation was significantly lower in HBV transfected cells during initial cell seeding. In
addition, the adhesion contact area of the normal HepG2 cell reached steady-state level of around $271 \mu^{2}$ at around 70 minutes. In contrast, $46 \%$ longer time was needed for HBV-replicating cells to reach the same steady state. The fact that cells transfected with the empty vector developed significant adhesion contact with an areas of $175 \mu \mathrm{~m}^{2}(43 \%$ of the steady-state value) at 20 minutes after cell seeding (Figure 4.14 panel C), supported that the observed adhesion contact evolution was not due to the transfection or the plasmid, but more likely related to the HBV replication.

The degree of cell deformation $(a / R)$ is a key biophysical parameter which collectively reflects the simultaneous spreading and contact formation of adherent cells on a planar substrate. In this study, it may be used as a geometry index for elucidating the influence of HBV replication on the responses of adherent cells. Figure 4.15 (A) showed the $a / R$ of normal HepG2 cells $(\odot)$, HepG2 cells transfected with the empty vector ( $\square$ ) and HepG2 cells transfected with $\mathrm{rHBV}(\mathbf{\Delta})$ on collagen-coated substrate. Each error bar represented the standard deviation of the data from at least 60 cells on four sample sets. Generally, $a / R$ of normal HepG2 cells rapidly increased against time during the initial five min of cell seeding (Figure 4.14 panel A). Similar trend was observed in cells transfected with the empty vector. In contrast, $a / R$ of HepG2 cells transfected with rHBV showed detectable level 10 min after cell seeding. The initial cell deformation rate for normal HepG2 cell and cell transfected with empty vector is 0.019 and $0.018 \mathrm{~min}^{-1}$, respectively. In contrast, the initial cell deformation rate of cells transfected with rHBV (calculated from 10 minutes onward) was at $0.013 \mathrm{~min}^{-1}$. Our results supported that the kinetics of cell deformation is dependent on HBV replication.


Figure 4.14:
A series of C-RICM images of (A) normal HepG2 cells, (B) HepG2 cells with transfected empty vector and (C) HepG2 cells transfected with rHBV, from 5 to 150 minutes after seeding on collagen coated glass coverslip at $37{ }^{\circ} \mathrm{C}$. The contact area was determined with the drawing tool of the software which indicates the area of contact between the cell and the substrate.

Cell adhesion to ECM or biomaterials is a highly dynamic process. The complex dynamical response of the adhesion energy between cell and ECM or biomaterial has been measured (Nicholas et. al., 2004, Cohen et. al., 2004). Contact mechanics model of adherent cell has been successfully used to determine adhesion energy of HepG2 cells (Yin et. al., 2003, Feng et. al., 2005). Figure 4.15 (B) showed the averaged adhesion energy of a population for normal HepG2 cells ( ) , HepG2 cells transfected with the empty vector ( $\boldsymbol{\square}$ ) and HepG2 cells transfected with rHBV ( $\mathbf{\Delta}$ ) against time of cell seeding on collagen coated substrate. The averaged adhesion energy of all types of cells spanned several orders of magnitude. The results indicated that normal HepG2 cells rapidly achieved a notable adhesion energy of $1.7 \times 10^{-15} \mathrm{~J} / \mathrm{m}^{2}$ at 5 minutes after seeding on collagen coated substrate. In contrast, the averaged adhesion energy of HBVreplicating HepG2 cells was negligible during the initial period of 19 minutes and was two orders of magnitude lower than that of normal HepG2 cells and cells transfected with empty vector at 20 minutes after cell seeding. Significant reduction in adhesion energy induced by HBV replication as discussed above remained after 90 minutes post cell seeding. Moreover, normal HepG2 cells and cells transfected with empty vector required 70 minutes to reach steady state in adhesion energy. HBV-replicating HepG2 cells take longest time of about 130 minutes in reaching a steady state of adhesion energy compared with the other two cell types. At steady state, the adhesion energy of all three types of cells approached the same level of around $1.2 \times 10^{-7} \mathrm{~J} / \mathrm{cm}^{2}$. It is possible that our observation was caused by the cytoskeleton alteration upon HBV replication which led to the change in the mechanochemical responses on ECM (Lara-Pezzi et. al., 2001b). Our
(A)

(B)


## Figure 4.15:

Adhesion Kinetics of HepG2 Cells transfected with rHBV genome. (A) shows the average degree of cell deformation $a / R$ of HepG2 cells infected with rHBV, HepG2 cells with transfected empty vector and normal HepG2 cells on collagen coated substrate against the time of cell seeding. Each error bar represented the standard deviation of the data from at least 60 cells on four identical samples. (B) The average adhesion energy of a population of HepG2 cells infected with HBV, HepG2 cells transfected with empty vector and normal HepG2 cell against time of cell seeding on collagen coated substrate. Data of at least 60 cells on four identical sample sets were used for the calculation of the average adhesion energy. (Cell treatment was performed and the analysis was carried out by Feng Zhiqin and A/P Vincent Chan, SCBE)

hypothesis that HBV might interfere with cytoskeletal changes was supported by the interaction between key cytoskeletal proteins and HBV associated proteins.

Furthermore, our findings on the delayed cell adhesion in HBV-replicating HepG2 cells have provided new insights on the mechanism of development of hepatocellular carcinoma, consistent with the observation that inhibition of cell adhesion is implicated in intrahepatic metastasis of human hepatocellular carcinoma (Genda et. al., 2000).

### 4.4.2 Focal Complex Formation Is Delayed in HBV Replicating Cells

The link between cytoskeleton reorganization, the reduction of adhesion rate and HBV replication was not clearly understood. It is widely known that integrin-mediated cell adhesion triggers the formation of focal adhesion complex through the association with actin cytoskeleton and subsequent clustering. In detail, focal adhesion sites compose of various cytoskeletal proteins such as vinculin, talin, and $\alpha$-actinin, and signaling molecules, including FAK, Src, and paxillin. The focal adhesion formation plays a critical role in the cell adhesion by serving as structural links between the cytoskeleton and ECM.

Vinexin $\beta$ is an adaptor protein that is involved in the process of actin polymerization (Kioka et. al., 2002). In a recent study, it had been shown that the SH3 domain of vinexin- $\beta$ binds to the hinge region of vinculin and vinexin localized with vinculin at the focal adhesion (Chen et. al., 2005). Moreover, the expression of vinexin- $\beta$
was found to enhance the formation of focal adhesion and cell spreading in 3T3 fibroblasts (Kioka et. al., 1999). Therefore the interaction of HBx with Vinexin following HBV replication could significantly reduce vinculin self-assembly necessary for focal complex formation. Fig. 4.16 (A) showed the confocal fluorescence images of normal HepG2 cells, HepG2 cells transfected with rHBV and HepG2 cells transfected with the empty plasmid immunostained with anti-vinculin at 20 minutes after cell seeding. The result showed that significant expression of vinculin was detected in a typical group of normal HepG2 cells (top panel) while vinculin was barely detected in the group of HBVreplicating cells (bottom panel). Specifically, dot-like structures of vinculin which have a diameter ranging from 0.5 to $1.5 \mu \mathrm{~m}$ emerged at the lamellipodium of normal HepG2 cells (Fig. 4.16 (A): single-cell image in top panel). The dot-like structures composed of vinculin are known as focal complexes which served as precursor of focal adhesion (Chen et. al., 2005). In contrast, only diffused vinculin instead of clusters was detected in the cytoplasm of HepG2 cells transfected by HBV (single-cell image in bottom panel Figure 4.16 (A). As a control, HepG2 cells transfected by empty vector also demonstrated the expression of similar focal complexes found in normal HepG2 cells (single-cell image in middle panel Figure 4.15 panel A). The result strongly supported that HBV replication delayed the formation of focal adhesion complexes during initial cell seeding. This delay of focal complex evolution coincides with the reduction of initial cell deformation rate and adhesion energy during the first twenty minutes of cell seeding. In particular, the formation of focal complexes serves as a traction base for cell movement, anchor point at the cell-cell connection, and as cement during morphogenesis (Cohen et. al., 2004).
(A)

(B)


Figure 4.16:
Immunofluorescence image staining for vincu(l)-3in of HepG2 ce(l)-3(l)-3s, HepG2 ce(l)-3(l) transfected wth empty vector and HepG2 ce(l)-3(l)-3s transfected wth rHBV. (A) Depicts the image taken 20 minutes post $c e(l)-3(l)-3$ seeding wil (B) depicts the image taken 90 minutes post ce(l)-3(l)-3 seeding (sca(l)-3mb)ara 200 minutes, there appear a de(l)-3ay in rHBV transfected HepG2 ce(l)-3(l)-3s in the formation of vincu(l)-3in patches, necessary for foca(l)-3 adhesion $\operatorname{comp}(\mathrm{l})$-3ex formation. This de(l)-3ay ws reflected in Figure 4.15. By 90 minutes, the ce(l)-3(l)-3s showd norma(l)-3 dense staining of vincu(l)-3in patches in a(l)-3(l)studied.

Towards 90 minutes of adhesion, the dot-like focal adhesion complexes in normal HepG2 cells and cells transfected by empty vector were transformed to dense patches at cell periphery (Figure 4.16 panel B). The elongated and oval vinculin containing structure had an averaged length of $4 \mu \mathrm{~m}$, generally known as focal adhesion (zoom-in view on the right of all panels). Interestingly, vinculin patches also emerged at cell periphery of HBV-replicating HepG2 cells after 90 minutes of cell seeding.

At the same time, all cell types demonstrated high level of diffuse vinculin in the cytosol. The formation of focal adhesion in HBV-replicating HepG2 cells may be caused by the integrin-collagen recognition and the subsequent cytoskeletal reorganization. The results further supported the transient effect of HBV replication on the adhesion energy and degree of deformation during the initial 20-30 minutes of cell seeding on ECM.

### 4.4.3 Delay in Cell Adhesion Is Mediated by SH3-binding Domain in HBx

To investigate the impact of interaction between vinexin- $\beta$ and HBx on cell adhesion process, HepG2 cells were transfected with either the wild type HBx or mutant HBx. The mutant HBx contained four proline to alanine mutations, in the SH3-binding motif. Cell adhesion analysis, similar to that carried out in this study on HBV-replicating cells, was then carried out.

Figure 4.17 shows the C-RICM images of HepG2 cell transfected by HBx, normal HepG2 cell and HepG2 cell transfected by mutant HBx (prolines mutation to alanines) during the initial stage of cell seeding on collagen coated coverglass. The result indicated
that the normal HepG2 cell and HepG2 cell transfected with mutant HBx develops an adhesion contact of 30.1 and $50.2 \mu \mathrm{~m}^{2}$, respectively, after 15 min of seeding. Interestingly, HepG2 cell transfected by wild type HBx failed to develop any strong adhesion contact during the initial 30 minutes of cell adhesion. After 36 min of seeding, the adhesion contact area of normal HepG2 cell and HepG2 cell transfected with mutant HBX reaches 122.4 and $142.7 \mu^{2}$, respectively. In contrast, the adhesion contact area of the HepG2 transfected with wild type HBx at 36 min is approximately $80 \%$ smaller than ( $30 \mu \mathrm{~m}^{2}$ ) that of normal HepG2 cell and HepG2 cell transfected with mutant HBx.

The observed delay in adhesion in cells transfected with the wild type HBX was similar to that observed in cells transfected with replicative genome (rHBV). Significantly, the importance of HBx protein in this cellular process was further indicated by the fact that mutations in the proline rich domain of HBx, identified in this study, resulted in the restoration of normal cell adhesion. Perhaps more importantly, our results suggested a direct involvement of interaction between HBx and vinexin $\beta$ via SH3 binding, as mutations at proline residues which have been known to be involved in SH 3 binding abolished the delay in cell adhesion.


Figure 4.17:
C-RICM images of (A) HepG2 cell transfected with wild type HBx, (B) normal HepG2 cell and (C) HepG2 cell transfected by mutant were incubated for 48 hour prior to trypsinization. They were then seeded onto collagen coated coverglass at the same time point. The initial stages of cell seeding on collagen coated coverglass, at 15 and 36 min respectively were documented. Note that HBx transfected cells resulted in a delay in cell adhesion as observed above and that this effect was restored when mutant HBx (proline rich region replaced with alanine) was transfected. White bar signifies $20 \mu \mathrm{~m}$.

Similarly to HepG2 cells transfected with rHBV, the average degree of deformation $(a / R)$ for HepG2 cell transfected by HBx was shown in Fig. 4.18 (A). The error bar of each data point represented the standard deviation of 60 cells on three samples. The result showed that $a / R$ of both normal HepG2 cell and HepG2 cell transfected by mutant HBx rapidly rose during the initial 30 min of seeding. On the other hand, $a / R$ for HepG2 cell transfected with HBx was undetectable until 36 minutes of seeding and remained lower than the value for both normal HepG2 cell and HepG2 cell transfected with mutant HBx from 36 to 120 min of seeding. After 80 min of seeding, $a / R$ of both normal HepG2 cell and HepG2 cell transfected by wild type HBx reached 0.83. Figure 4.18 (B) showed the average adhesion energy for HepG2 cell transfected by HBx, normal HepG2 cell and HepG2 cell transfected by mutant HBx against the time of cell seeding on collagen coated coverglass. The result showed that adhesion energy of both normal HepG2 cell and HepG2 cell transfected with wild type HBx spanned eight orders of magnitude between 8 min and 2 hour of seeding. In contrast, the adhesion energy of HepG2 cell transfected by wild type HBx was not detectable until 36 min of seeding and was seven orders of magnitude lower than that of normal HepG2 cell and HepG2 cell transfected by mutant HBx.
(A)

(B)


Figure 4.18:
Adhesion Kinetics of HepG2 Cells Transfected with HBx. (A) shows the average degree of deformation $(a / R)$ for HepG2 cell transfected by HBx, normal HepG2 cell and HepG2 cell transfected by mutant HBx against the time of cell seeding on collagen coated coverglass. (B) The average adhesion energy for HepG2 cell transfected by HBx, normal HepG2 cell and HepG2 cell transfected by mutant HBx against the time of cell seeding on collagen coated coverglass. Compared to Figure 4.15, HBx transfected cells resulted in a higher degree of deformation compared to rHBV transfected HepG2 cells. Also, a stronger decrease in adhesion energy was observed when transfecting HepG2 cells with HBx compared to rHBV indicating that HBx might be directing these differences of rHBV. Mutant HBx (prolines replaced with alanines) transfected cells showed no difference compared to HepG2 cells alone. (Cells treatment was performed and the above analysis were made by A/P Vincent Chan, SCBE)

### 4.5 Discussion and Conclusion

It is noticed that hepatocyte cell lines including HepG2 and Huh-7 cells are able to support HBV replication (Paran et. al., 2001) and hence provide a cell-base experimental model for HBV research. Our previous results suggest that the replicative genome delivered through transfection was able to produce significant amount of HBV particles (Toh et. al., 2005). In this study, the effect of HBV replication on cell adhesion has been explored with our recently developed cell-based HBV replication system in combination with real-time biophysical measurements.

It has been demonstrated that the lag time of adhesion contact evolution of HepG2 cells with HBV replication is significantly increased by two times compared to that of normal HepG2 cell on collagen coated substrate. During the initial twenty minutes of cell seeding, only diffuse forms of vinculin was detected in HBV replicating cells while vinculinassociated focal complexes were found in normal and control cells. Similar delay in cell adhesion in HBV-replicating cells was observed in cells transfected with HBx, the smallest HBV protein, suggesting its involvement in this cellular process. In addition, a proline rich region found in many SH 3 binding proteins was identified in $\mathrm{HBx} . \mathrm{HBx}$ was found to interact with the focal adhesion protein, vinexin $\beta$, through the SH 3 binding. Using only HBx overexpression, we have found that the loss of adhesion energy was even higher then that of HBV transfection. This could be due to the relative amount of HBx protein expressed by replicating HBV compared to direct expression of the HBx. Notably, this delay in adhesion in the initial cell seeding was not observed upon the use
of a proline-alanine mutant of HBx as described earlier. Our results suggest that HBx is involved in the cytoskeletal reorganization in response to HBV replication.

Taken together, our results indicated that the expression of wild type HBx significantly dampened the kinetic of adhesion contact evolution. Such a delay in cell adhesion was not observed by transfection of HBx with specific mutations in the SH 3 binding motif. It is likely that our identified interaction between vinexin $\beta$ and HBx played a direct role in the cell adhesion process in the context of HBV replication.

The temporal trends of degree of focal adhesion formation and adhesion energy of the cells are found to be perturbed by HBV replication during the initial stage of cell seeding on collagen coated substrates. Most importantly, the biological origin of our observed HBV induced responses is also suggested by the characterization of interaction between vinexin $\beta$ and HBx , via a newly identified SH3-binding motif in the latter.

HBV and HBx result in cytoskeletal changes previously undescribed. We also observed that replicative genome transfected cells displayed perturbations at the cellular periphery. Therefore, we proceeded to further explore on how replication of HBV can cause changes in cytoskeletal reorganization.

Acknowledgement: C-RICM, cell deformation and adhesion energy experiments were performed in conjunction with Dr Feng Zhiqin (SCBE, NTU). Cells for the analysis were prepared by me while data analyses were performed by her.

## Chapter 5 <br> HBV and Rho GTPase

### 5.1 HBV and Rho GTPase

Hepatitis B Virus (HBV) is a causative agent for various severe liver diseases such as liver cirrhosis and hepatocellular carcinoma. As the search for the cellular receptor of HBV remains elusive, the understanding of its interactions upon viral entry, with cellular proteins are crucial in the elucidation of the downstream cascade of events leading to disease progression. Therefore, understanding of the process of HBV attenuation of cellular process is important in a concerted effort for drug intervention to minimize their disease-causing effects. As our previous studies have implicated cytoskeleton attenuation as a potential part of HBx interfering with cellular pathway, it is likely that HBx might also act via another pathway in interfering with cellular movement or transformation potentially leading to cellular malignancy.

### 5.1.1 Diseases and Rho GTPase

It is known that viral replication is an intricate process requiring specific interactions between the virus and host's cellular proteins allowing the virus to make use of the cellular machinery efficiently for their viral protein production and packaging. The elucidation of cellular responses upon viral infection is critical for designing effective antiviral therapy. Viral infections have been shown to result in filopodia and lamellipodia formation for triggering cell migration as a way of infection defense (Laakkonen et. al., 1998 , Lee $\& \mathrm{Ng}$, 2004) and deregulation of this normal cellular mechanism might lead to malignancy onset. The formation of these structures are mainly regulated by Rho family of small GTPases, including Cdc42, Rac1 and RhoA. Rho GTPases, which have been known to regulate several cellular functions such as adhesion, polarization, spreading,
migration, gene expression, endocytosis as well as cell growth. Specifically, Cdc42 induces filopodia formation to regulate cell polarity while Rac1 induces membrane rufflings and lamellipodia formation. RhoA induces formation of stress fibers (Manser et. al., 1997, Hall, 1998, Giancotti and Ruoslahti, 1999, Price et. al., 1998). Indeed, Cdc42 and Rac1 activation have been linked to increased HIV-1 replication (Lu et. al., 1996). Also, regulation of Rac1 and Cdc42 activation have been found to be critical in early Herpes simplex virus type I infection and in particular, dominant-negative Cdc42 resulted in drastic decrease in HSV-1 viral infectivity (Hoppe et. al., 2006). It is hence not surprisingly that viruses can make use of host cytoskeletal control modules in an attempt to control their replication or viral protein trafficking.

Activation and dissociation of Rho GTPases are controlled by guanine nucleotide exchange factor (RhoGEF) and GTPases activating protein (RhoGAP) respectively. Targeting of the Rac1 have been known to be controlled by various Guanine nucleotide exchange factor (GEF). Rac1, one of the important GTPase is found mainly as an inactive bound form to RhoGDI. Activation usually involves the dissociation of the inhibiting GDI complex and this have been linked to calcium signaling (Price et. al., 2003). In particular, it has been shown that $\beta$ PIX is critical for targeting of the (1) the intracellular targeting of Rac1 as well as (2) activation of Rac1 resulting in its downstream signaling pathway as well as morphological presentation (ten_Klooster et. al., 2006).

In the present study, we applied various microscopic and biochemical techniques to understand both at the nano-scale morphological changes as well as the potential interaction of the HBV replication with respect to small GTP-binding proteins.

### 5.1.2 HBV Replicating Cells Show Morphological Changes

A cell-based system for HBV replication using a linearized form of HBV genome cloned into mammalian expression vector, pCDNA3.1+ have been established (Chen et. al., 2000, Tan et. al., 2006, Lu et. al., 2006). Transfection of the constructed genome into mammalian cells have been previously shown to release HBV viral particles (Toh et. al., 2005, Pan et. al., 2005) and made used of in this study to understand the morphological and biochemical changes of HBV replication. For safety issues, HepG2 cells transfected with HBV replicative genome (rHBV) were fixed prior to observation. It has been shown that the morphology of the fixed cells only undergo minor change compared to that of intact cells (Lee \& Ng, 2004). Figure 5.1(A) shows an AFM topographic image (A) and an amplitude image (B) of a typical HepG2 cell ( $50 \mu \mathrm{~m} \times 50 \mu \mathrm{~m}$ in area). In the absence of HBV replication, the contour of the adherent cells was smooth with minor protrusions (white arrow) as observed in (C). In contrast, HBV replicating HepG2 cells show perturbations to the cell periphery as evidenced by Figure 5.1(B). Figure (A) and (B) shows the topographic and amplitude image respectively. As seen in the zoomed-in view, cells showed membrane rufflings (white arrow) and evidence of lamellipodia formation giving a slightly "spiky kind" of cell morphology. As these cellular morphological changes are reminiscent of the cellular effects caused by cellular Rho GTPases (Hall, 1998), it prompted us to investigate HBV replication to cellular perturbations through
(A)

(B)


Figure 5.1:
AFM study of normal (A) pCDNA3.1+ transfected HepG2 and (B) pCDNA3.1+ HBV replicative genome (rHBV) transfected HepG2 cells. Panels A and B of each shows the amplitude image of the transfected HepG2 cell (Scan area is $50 \mu \mathrm{~m} \times 50$ $\mu \mathrm{m}$ ) while $C$ show the zoom-in view of a height image of the red-boxed area with scan size of $10 \mu \mathrm{~m} \times 10 \mu \mathrm{~m}$. As observed, (B) HBV replicating HepG2 cells show perturbations at the cellular periphery compared to (A) empty vector transfected HepG2 cells. (AFM analysis were performed by Dr Fang Ning (SCBE, NTU)
these Rho GTPases such as Rac1, Cdc42 and RhoA. Furthermore, it has been shown recently that HIV-1 infection resulted in enhanced adhesion of T-cells possibly through RhoA activation (Takano et. al., 2007) further substantiating the potential of viral interaction with host molecules attenuating cytoskeleton modification. As the cellular receptor for HBV viral entry remain elusive, the understanding of the cellular response upon viral entry and replication will be critical for development of effective anti-viral intervention strategy.

### 5.1.3 Rho GTPases

Rho GTPases acts as switches in controlling signaling transduction cascades in all mammalian cells. Cycling between an "active" GTP-bound and "inactive" GDP-bound form with inherent GTPase activity, they control downstream signaling. Belonging to the Ras superfamily of small GTPases, they are further divided into many sub families with Cdc42, Rac1 and RhoA studied most intensely (Hall, 2005). Activated Cdc42, Rac1 and RhoA are known to induce cytoskeletal changes, consisting of actin protrusions such as filopodia, lamellipodia / membrane rufflings and stress fibers respectively (Price et. al., 1998). These Rho GTPases are maintained in the "inactive" GDP-bound form by RhoGDI (guanine nucleotide dissociation inhibitors). They are activated in requirement by Rho GEF (Guanine nucleotide exchange factor) and inactivated by Rho GAP (GTPase activating protein). Maintenance of balance of activations and inactivations are important as Rho GTPases control intricate cellular events such as cell migration, chemotaxis, cell contraction, phagocytosis and regulation of cell cycle progression (Etienne-Mannville and Hall, 2002). Hence any deregulation may result in deleterious effects.

### 5.1.4 Morphological Changes of HBV Replicating Cells Similar to Rac1 V12 Transfected Cells

Actin cytoskeletal changes are brought about by the Rho family of small GTPases, Cdc42, Rac1 and RhoA. Activated forms of each of these small GTP-binding proteins results in differential cellular perturbations at the cell periphery through actin polymerization. Previous reports have shown that activated forms of the above Rho GTPases effects filopodia, membrane rufflings / lamellipodia and stress fibers respectively (Hall, 1998). To further understand the phenomenon observed by AFM, we performed phalloidin staining of actin filaments.

To this end, we made use of constitutively active (V12) and negative mutants (N17) of Rac1 and Cdc42, V12 and N17 respectively (Manser et. al. 1998, Koh et. al., 2001). They were transfected individually into HepG2 cells in parallel with HBV replicative genome and pCDNA3.1+ (as mock control). High transfection efficiency of HepG2 cells were achieved by the use of Nucleofector (Amaxa, GmBH) using specialized Solution V. High efficiency protocol T-28 were made use of to ensure high transfection efficiency. Figure 5.2 shows (A) phase contrast image with the corresponding (B) fluorescent microscopy for pDSRed (Clontech) expression in HepG2 cells by nucleofection. As observed, an average of $\sim 70 \%$ transfection efficiency was achieved.

After high-efficiency nucleofection of (1) pCDNA3.1+ empty vector, (2) pCDNA3.1+ HBV replicative genome, (3) pXJ40HA-Rac1 V12 (activated Rac1 mutant) and (4) pXJ40HA-Cdc42 V12 (activated Cdc42 mutant), the cells were immunostained with phalloidin tagged with Alexa Fluor 488nm (Invitrogen Inc., USA) for visualizing the actin filaments of the cytoskeleton. Repeated transfections and image capture were performed and documented in figure 5.3. As per literature (Hall, 1998), activated mutants Rac1 V12, Cdc42 V12 and RhoA V14 transfected HepG2 cells displayed their respective phenotype as shown in panel of triplicates (Figure 5.3 panels A, B and C). A clearer representation was obtained by a crop of the cell periphery individually (Figure 5.3 panel D). Perturbations to the cellular periphery were observed in higher magnification in HBV replicating cells showing ruffling-like appearances compared to the smooth mock (pCDNA3.1+) transfected cells. Comparatively, Cdc42 V12 transfected cells showed longer protrusions equivalent to the filopodia while Rac1 V12 transfected cells showed shorter but more intense membrane rufflings as shown by the cropped pictures (Figure 3D). RhoA V14 on the other hand showed significantly different presentation with strong staining of the stress fibers, compared to Rac1 and Cdc42.

As observed in Figure 5.3 (D), HBV replicating cells and Rac1 V12 transfected cells showed ruffles-like structures on their cell surface, reminiscent of the membrane rufflings and filopodia caused by activated Rac1. There may hence be a possibility that HBV viral replication can activate Rac1 resulting in activated Rac1 morphology of HBV replicating cell. It would also be interesting to understand the effects of activated Rho GTPases on HBV replication.

### 5.2 Activation of Rho GTPases Increases Viral Replication

### 5.2.1 Activated Rac1 and Cdc42 Increases HBV Viral Replication

Notably, HIV-1 viral replication was previously reported to be highly dependant on Cdc42 and Rac1 activation (Lu et. al., 1996). Perturbed by the microscopic observations, we next seek to understand if Rac1 and Cdc42 activation also plays a direct role in HBV viral replication. We established the use of a Real-time RT-PCR on the preCore region of HBV, indicative of HBV pgRNA, product of a critical first step in viral replication (Seeger \& Mason, 2000).

As depicted in Figure 5.4, activated mutants of (A) Rac1 and (B) Cdc42 cotransfection with HBV replicative genome (rHBV) resulted in increased viral pgRNA production compared to rHBV transfection alone. Notably, activated Cdc42 showed the highest fold increase in viral intermediates ( $\sim 2.5$ fold) compared to $\sim 1.4$ fold increase by activated Rac1 co-transfection with rHBV. However, in the dominant negative N17 mutants, Rac1 showed a decrease to $\sim 0.6$ fold viral pgRNA production. Cdc42 N17 on the other hand showed slight increase over rHBV alone. Besides, activation of RhoA V14 also resulted in no significant changes in viral replication while RhoA N19 showed decrease to $\sim 0.6$ fold. Therefore, activation of these Rho GTPases, Cdc42 and Rac1 led to increased viral replication and HBV might harness this activation in true context for increased viral genome production. HBV could potentially increase their replication through the increase in actin reorganization potentiating the export of viral particle.

## (A)

(B)


Figure 5.2:
This figure shows the transfection efficiency of HepG2 cells using the Amaxa Nucleofector technology. We probe the transfection efficiency by transient transfection of pDSRed (Clontech) vector alone. (A) Depicts the phase contrast image obtained 24 hours after transfection while (B) shows the corresponding expression of the red fluorescence detected. The image was digitally acquired using an Olympus IX71 fluorescent microscope and showed transfection efficiency of approximately 70 \%.


Figure 5.3:
Figure above shows the fluorescent microscopy pictures of HepG2 cells transfected with (1) pCDNA3.1+ (empty vector), (2) rHBV (3) Rac1 V12 (4) Cdc42 V12 and (5) RhoA V14 using Amaxa Nucleofector technology and immunostained with Phalloidin - Alexa fluor 488nm for actin filaments. Rows (A), (B) and (C) shows the triplicates of images taken from individual transfection while (D) depicts a representative cropped view of the cell periphery from each of the transfection. HBV replicative genome shows increased ruffling and presence of lamellipodia presentation comparable to that of dominant active Rac1 V12 phenomenon but to a lesser extent. On the other hand, dominant active Cdc42 V12 and RhoA V14 present physically different presentations of the filopodia and stress fibers respectively. pCDNA3.1+ acts as a negative control for HBV replicative genome transfection and shows the typical staining of HepG2 with enhanced actin at the periphery of the cells. (White bar within each picture represents length of $\mathbf{4 0} \boldsymbol{\mu \mathrm { m }}$ )

### 5.2.2 HBV Specifically Activates Rac1

Since activated Rac1 and Cdc42 resulted in increased viral replication as evidenced by increased pgRNA production, we next sort to understand if HBV plays a role in the activation of these Rho GTPases. To achieve this, we made use of Cdc42 / Rac1 interactive binding (CRIB) domain of PAK1 which binds specifically to activated forms (GTP bound) of Rac1 or Cdc42 (Price et. al., 2003). Dominant active (V12) and inactive mutants (N17) of Rac1 and Cdc42 were made use of as positive and negative controls in this experiment. pCDNA3.1+ (empty vector) and HBV replicative genome (cloned in pCDNA3.1+) were co-transfected with Rac1 WT and Cdc42 WT individually to follow as to whether they are activated by HBV replication. GST-PAK 1-207aa (containing the CRIB domain) were expressed and used for the precipitation of activated Rac1 / Cdc42. As observed in Figure 5.5 (A), constitutively inactive and active mutants showed their respective pulldown phenomenon detected by Western analysis using anti-HA, with Rac1 V12 and Cdc42 V12 binding to the GST-PAK 1-207aa, but little or no binding to their N17 counterparts in accordance with previous findings (Price et. al., 2003).

HBV replicating cells co-transfected with Rac1 WT, showed positive binding of the Rac1 to the PAK 1-207aa, indicative of an activated Rac1-GTP form. Contrasting this, the pCDNA3.1+ (empty vector) co-transfected cells did not show any activation of Rac1 WT as evidenced by non-binding to the CRIB (Figure 5.5 panel A). On the other hand, Cdc42 WT co-transfected with either pCDNA3.1+ or HBV replicative genome (rHBV) showed that neither resulted in activation of Cdc42 WT to Cdc42-GTP state as Western analysis proved negative (Figure 5.5 panel A). Therefore, we have shown that HBV


Figure 5.4:
Real-time RT-PCR for viral replication intermediates after co-transfection with various Rho GTPase mutants. The figure depicts the fold increase of rHBV viral replication from the various GTPases co-transfected with rHBV. The results from the various transfections were normalized by the use of actin concurrently and are the results of 3 replicates of transfection. Fold increase were calculated with respect to rHBV alone transfected cells (tabulated as 1 fold). As observed, Cdc42 V12 cotransfected with rHBV gave the highest fold increase followed by that of Rac1 V12 co-transfected cells.
replicating cells showed an activation of Rac1 specifically and that this activation might potentially have some effects on HBV viral replication. Surprisingly, Cdc42, an upstream activator of Rac1 was not activated. Hence, HBV might act downstream of the typical activated Cdc42 to activation of Rac1 pathway.

### 5.2.3 HBV Replication Signals Through Rac1 Activation

HBV activation of Rac1 remains to be understood. It has been shown that Rac1 activation is required for maintenance of malignancy in mouse skin tumor cells through the activation of mitogenic signaling pathways such as ERK1/2 and AKT activation occurs downstream of Rac1 activation (Kwei et. al., 2006). Using phosphorylated antibodies in Western blot analysis, we have shown that HBV replication resulted in phosphorylation of ERK1/2 as well as AKT similar to previous reports (Chung et. al., 2004). ERK1/2 and AKT phosphorylation were also observed in Rac1 V12 transfected cells (Figure 5.5 panel B) and could potentially explain a role of Rac1 activation by HBV replication resulting in the downstream MAPK and PI3K/AKT activation. There remains a potential of a direct HBV activation of Rac1 resulting in their downstream phosphorylation of effector molecules.
(A)

(B)


Figure 5.5:
(A) The CRIB binding assay utilizing GST fusion protein of PAK1-207aa which contains the CRIB domain that binds specifically to only GTP bound Rac1 and Cde42 (activated forms). The left panel depicts the binding assay results from Rac1 binding assay of HBV replicative genome transfected compared to the dominant active (Rac1 V12) and dominant negative (Rac1 N17) controls by Western analysis using mouse monoclonal anti-HA antibodies (Santa Cruz Biotech). Bottom shows the loading consistency of the corresponding binding protein, GST-PAK1-207aa by Coomassie Blue staining of the SDS-PAGE after Western transfer. Concurrently, the right panel shows the binding assay results from Cdc42 binding assay of HBV replicative genome transfected compared to the dominant active (Cdc42 V12) and dominant negative (Cdc42 N17) controls.
(B) shows the phosphorylation status of proteins extracted from rHBV-transfected and that of activated Rac1 V12-transfected cells. Phosphorylation antibodies for ERK1/2 (BD Transduction laboratories) and AKT (Cell Signaling Technology) were made use of for the immunoblots. Total ERK1/2 and AKT were respectively immunoblotted for loading consistency. Immunoblots using anti-HA in the bottom most panel was used to detect for the presence of the Rac1 V12 transfection.

### 5.2.4 HBV Activates Rac1 Independently of Upstream Cdc42

It has been previously shown that Cdc42 activation leads to Rac1 activation followed by downstream cascade of events leading to morphological changes (Price et. al., 1998, Obermeier et. al., 1998). As shown by the Figure 5.4, activation of Rho GTPases resulted in increased viral replication. Interestingly, inactive mutant of Cdc42 still showed increased HBV replication albeit being lower then the activated forms. Coupled with the specificity of Rac1 activation by HBV replication as observed in Figure 5.5, it seems that HBV might act directly on Rac1 independently of upstream Cdc42. To further understand this, we co-transfected rHBV with Cdc42 V12 and Rac1 N17 to mimic the knockdown of its known downstream Rac1 activation, and observe its effects on HBV replication. As a control, rHBV was co-transfected with Cdc42 V12 and pXJ40HA (empty vector). Basal level of HBV replication was obtained by transfection with rHBV alone.

Indeed, as shown in Figure 5.6, rHBV + Cdc42 V12 + pXJ40HA transfected cells (lane 1) showed increased viral replication, in a level similar to that of transfection with Cdc42 V12 alone (Figure 5.4). Interestingly, Rac1 N17 co-transfection with Cdc42 V12 and rHBV (lane 2) resulted in a decreased viral replication rate close to but lower then the level of rHBV alone transfection (lane 3). Hence, it could be deduced that (1) Activation of Cdc42 leads to the downstream increased HBV replication through Rac1 activation and (2) Coupled with the CRIB


Figure 5.6:
Real-time RT-PCR was used to analyze the potential of rHBV acting downstream of Cdc42 activation. Cdc42 V12 co-transfected with rHBV showed increase viral replication intermediate while co-transfection with Rac1 N17, which mimics a knock down of its activation function resulted in decreased viral yield compared to rHBV alone transfected cells.
binding assay, HBV might indeed result in increased Rac1 activation leading to increased HBV replication independent of upstream Cdc42 activation. The higher level of viral replication by activated Cdc42 compared to activated Rac1 (Figure 5.4) can be explained by the more efficient cellular signaling mechanism through a Cdc42 activation-Rac1 activation pathway. Taken together, HBV activates Rac1, independently of upstream Cdc42 and this remains to be examined.

### 5.3 TranSignal SH3 Domain Array I for Identifying Protein Interaction

### 5.3.1 HBx Shows Similar Membrane Perturbation of HepG2 Cells

Next, we sort to understand if HBx might affect cellular morphology in a fashion similar to HBV replication. HBx have been previously shown to interact with Vinexin $\beta$ in Chapter 4 and affect cellular adhesion, mediated by the proline-rich segment binding to SH3 domains. Hence, it might not be surprising that HBx can interact with other SH3 domain-containing proteins that attenuate other cellular signaling pathway(s). With that, we transfected pEGFP-HBx FL into Huh7 liver cells. Concurring with previous results, HBx shows the presence punctuated green dots around the peri-nuclear region (Figure 5.7 (A) and (B) panel i). The coverslip was subjected to staining with Phalloidin tagged with Alexa fluor 546 nm for visualization of the actin filaments and represented in Figure 5.7 panel ii. Figure 5.7 panel iii shows the superimposed image of panel i and ii. Within the same panel, cells transfected with HBx and those without were chosen and region was selected for further study. In panel ii, white box represents the transfected cells while yellow box represents the untransfected cells within the same pool. As observed in duplicate results depicted in Figure 5.7 (A) and (B), HBx transfection consistently


Figure 5.7: HBx transfection present similar membrane perturbations observed in rHBV replicating cells. (A) and (B) above shows duplicates the transient transfection of pEGFP-c1-HBx full length into HepG2 cells using Amaxa Nucleofector. After transfection, cells were fixed with $3 \%$ Paraformaldehyde and stained with phalloidin- Alexa fluor 546nm (Molecular probe) specific for actin filaments. HBx displayed localization in the perinuclear region as previously reported as shown in panels (i) \& (iii). HBx transfected cells displayed ruffling like behaviour similar to that of rHBv transfected cells as depicted in panels (ii) \& (iv) (boxed in white). Comparatively, cells within the same treatment not transfected with the GFP were also compared as negative control (ii) $\mathcal{\&}$ (iv) (boxed in yellow). White bar signifies $50 \mu \mathrm{~m}$.
resulted in a phenotype similar to rHBV replicating cells on the surface with ruffling like appearance as previously discussed in Section 5.1.3. We then proceed to explore the possibility of HBx interaction with cellular proteins resulting in the above mentioned phenomena.

### 5.3.2 Potential Mechanism of Rac1 Activation by $\boldsymbol{\beta P I X}$ Through Interaction with

## HBx Proline-rich Region

The hepatitis B virus $X$ protein ( HBx ) has been previously shown to be important for HBV viral replication (Yen, 1996) amongst its many other functions, we have shown the presence of proline-rich "PXXP" motif within HBx protein (Tan and Chen, 2005) and showed its importance in mediating cytoskeletal changes through interaction with cellular protein, vinexin $\beta$ (Tan et. al., 2006). This proline-rich region was previously cloned as a GST fusion and expressed successfully with a 6 X Histidine tag (Figure 5.8 panel A). Using this proline-rich motif (Figure 5.8 panel A) cloned with 6 X HIS interaction with TranSignal SH3 domain array I (Panomics, USA), we have identified amongst others, $\beta$ PIX, a RhoGEF (Figure 5.8 panel B). Similar to our previous study, c-Src showed positive binding while GST served as the negative control (Figure 5.8 panel B). A list of the various other interacting proteins of HBx proline- $\mathrm{r}^{\circ}$ ch construct was listed in Figure 5.8 (C).
(A)

(B)

$\begin{array}{llllllllll}1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10111213141516171819202122\end{array}$
(C)

| Column <br> $(\#, \#)$ | ID | Proteins Interacted |
| :--- | :--- | :--- |
| $\mathrm{A}(7,8)$ | Cortactin | Cortactin |
| A $(11,12)$ | YesI | Yamaguchi Sarcoma virus oncogene homolog 1 |
| A (19,20) | CRK-D2 | Avian Sarcoma virus CT10 oncogene homolog, domain \#2 |
| B (11,12) | c-Src | Cellular Rous Sarcoma viral oncogene homolog |
| $\mathrm{C}(5,6)$ | Y124 | PAK-interacting exchange factor beta |
| $\mathrm{C}(7,8)$ | PEXD | Peroxisomal membrane proteins |
| $\mathrm{D}(3,4)$ | Abl | Abelson tyrosine kinase |
| $\mathrm{D}(13,14)$ | ITSN-D2 | Intersectin1, SH3 domain \#2 |

Figure 5.8:
HBx proline-rich probe on TranSignal SH3 domain Array I.
(A) Constructs of proline-rich region of HBx (GST-PXXP-HIS) were expressed with 6xHIS tag and used to probe for interaction with the SH3 domains. The top panel shows the immunoblot using anti-GST while bottom panel shows the specific immunoblot with anti-HIS for the incorporated C-termini 6 X HIS tag.
(B) depicts the TranSignal SH3 domain array I after immunoblotting using the GST-PXXP-HIS construct and detected with anti-HIS antibodies provided. Essentially, HIS only peptide serves as controls within this blot and occupy the extreme right row of duplicates $A, B, C$ and $D(21,22)$ as well as the bottom most horizontal role $\mathbf{E}(\mathbf{1}$ to 20 ). SH3 domains of various known proteins were spotted in duplicates (Positive result is only upon detection of signal from both spots) on the membrane. As highlighted, c-Src B $(11,12)$ showed positive binding while GST D $(17,18)$ acts as negative control. Other interesting proteins identified through this blot include cortactin (cortical actin binding protein) A (7,8) and $\beta$ PIX (RhoGEF) C (5,6).
(C) List of proteins that interact with TranSignal SH3 domain array I.

### 5.3.3 Interaction of HBx Proline-rich Region with PAK-Interacting Exchange Factor Beta ( $\boldsymbol{\beta P I X )}$

To further confirm this interaction, we expressed mammalian form of $\beta$ PIX (Koh et. al., 2002) in HEK293T cells through transient expression (Figure 5.9 panel A) as evidenced by the detection of the FLAG tag using Western with anti-FLAG M2 (Sigma). A GST pulldown assay (Figure 5.9 panel B) utilizing this mammalian expressed $\beta$ PIX was conducted. Essentially, GST (negative control), GST-PAK1 1-207aa-FLAG (positive control as it contains the binding domain for $\beta$ PIX), GST- HBx PXXP and GST- HBx AXXA (constructed through site-directed mutagenesis as seen in previous work (Tan et. al., 2006), constructs were used for this pulldown assay. Negative binding was observed for the GST construct as negative control. As expected, positive binding to the PAK1 1207 a.a.-FLAG protein was shown by the detection of the $\beta$ PIX though a FLAG tag (Note: GST-PAK 1-207aa is also FLAG tagged). Interestingly, HBx PXXP fragment showed binding of $\beta$ PIX similar to PAK1 and this binding was reduced drastically by the HBx AXXA fusion protein. Hence, HBx may have a direct interaction with $\beta$ PIX through its proline-rich region.

To understand the intracellular effects, we perform cellular immunostaining to see if there is co-localization of HBx with $\beta$ PIX. Basically, pEGFP-C1-HBx was cotransfected with pXJ 40 FLAG- $\beta$ PIX. Immunostaining was performed against $\beta$ PIX using monoclonal anti-FLAG (M2) antibodies (Sigma Aldrich) and then detected by goat antimouse tagged with Alexa fluor 546nm (red). pEGFP-C1 vector alone was co-transfected with pXJ40FLAG- $\beta$ PIX as a control. As depicted in Figure 5.10 (B), duplicates of GFP-

HBx transfected Huh7 cells showed the staining surrounding the nucleus. $\beta$ PIX, stained red by Alexa fluor 546 nm showed co-localization as observed by the yellow spots of HBx localization. In contrast, duplicates of pEGFP-C1 alone showed no significant colocalization (Figure 5.10 panel A). This further shows the interaction between HBx and a RhoGEF, $\beta$ PIX and might explain the effects of HBx on signaling transduction pathways leading to downstream effects. HBx is found predominantly in the cytoplasm (as observed in Figure 5.10 panels A and B) and it has also been shown to be Localized to the proteasome (Chen et. al., 2001b) as well as mitochondria (Henkler et. al., 2001, Takada et. al., 1999).
$\beta$ PIX, a RhoGEF was previously identified to be a binding partner of Pak1 (Bagrodia et. al., 1998, Manser et. al., 1998) and plays a role in Rac1 activation downstream of activated Cdc42 (Obermeier et. al., 1998, Klooster et. al., 2006). Hence, it is possible that HBV replicating cells act through the HBx protein interaction via its proline-rich motif with $\beta$ PIX. Activation of GEF activity may hence lead to increased GTP forms of Rac1, potentiating increased viral replication. HBV replication-induced activation of Rac1 could also potentially lead to a change in actin cytoskeleton resulting in transformation of liver cells, potentiating the development of HCC. As such, elucidation of potential induction pathway might be important in the therapeutic strategy for HBV carrier, to protect them against liver diseases. Figure 9 depicts the potential pathway through which HBV might activate Rac1, independently of Cdc42 activation resulting in downstream cytoskeletal changes and increased viral replication.
(A)

(B)


Figure 5.9:
Expression of $\boldsymbol{\beta P I X}$ and GST immunoprecipitation assay.
(A) depicts the mammalian expression of $\beta$ PIX transfected transiently in HEK293T cells compared to empty vector, pXJ40FLAG detected using anti-FLAG M2 (Sigma). Corresponding consistency in loading was shown in the bottom panel using anti $\beta$ actin antibodies (Sigma). (B) shows the GST pulldown assay to confirm the binding of HBX proline-rich region (GST-PXXP) to full-length mammalian expressed $\boldsymbol{\beta P I X}$ (Lane 3). Mutant proline-rich region of HBX with (prolines replaced with alanine), designated GST-AXXA, showed highly decreased binding to the $\beta$ PIX (Lane 4) while GST acts as the negative control. GST-PAK 1-207aa acts as the positive control and confirmed the binding of $\boldsymbol{\beta P I X}$ to PAK $\mathbf{N}$-terminal as previously shown (Koh et. al., 2002).


Figure 5.10:
Colocalization of HBx and $\beta$ PIX
(A) Duplicate results depicting the immunostaining of pXJ40FLAG- $\beta$ PIX and pEGFP-C1 (Clontech) co-transfected Huh7 cells fixed by paraformaldehyde and immunostained with anti-FLAG and secondary antibody conjugated to Alexa 546nm. Green shows the GFP localization was diffused while red shows the localization of the $\beta$ PIX. Comparatively, (B) shows the duplicate results of pEGFP-C1-HBx co-transfection with pXJ40FLAG- $\beta$ PIX. Colocalization can be observed in the merge image of yellow spots surrounding the nucleus. White bar signifies $50 \mu \mathrm{~m}$.

### 5.4 Discussion and Conclusion

Increasing evidences have shown that Rho GTPases play important roles in regulating cytoskeletal remodeling such as formation of filopodia, membrane rufflings, and lamellipodia and stress fibers. They are hence are involved in a myriad of cellular signaling pathways and also play important role in cell migration and other important cellular events (Hall, 1998). These small GTP-binding proteins are controlled by carefully orchestrated events in the cells, essentially through the use of regulatory proteins including Guanine nucleotide exchange factor (GEF) and GTPase activating protein (GAP) to ensure homeostasis and balance (Etienne-Manneville and Hall, 2002). It is important to point out that deregulation of this cycle could have drastic deleterious effects, leading to cancer development. In the case of liver diseases, a particular example is the decrease in cell proliferation as well as invasive characteristics of HCC in patients with tumor suppressor gene DLC1 (Deleted in liver cancer) coding for a GAP (Wong et. al., 2005).

As observed by Diao et. al. in 2001, HBx may be involved in various signal transduction pathways potentially, thus affecting the downstream disease progression. Indeed, continued activation of signaling transduction may perturb normal cell cycle and cell morphology potentially, contributing to cancer development. Our report has established a potential role of HBV replication in maintaining Rac1 activation. In addition, HBV replication seems to be involved in the activation of Rac1 specifically and that this activation resulted in increased HBV viral replication. Increase in viral replication by Rho GTPases have been shown previously in HIV-1 (Lu et. al., 1996) but
not for HBV to date. Recently, it has been shown that the attenuation of Rho GTPases is implicated in controlling HSV-1 infectivity (Hoppe et. al., 2006). Hence, it is likely that HBV might affect this key cellular signaling and regulatory pathway leading to its downstream liver disease progression. In addition, activation of Rac isoforms have also been previously reported in various cancers and was shown to be involved in the metastases of breast carcinoma cells (Baugher et. al., 2005).

Using an established cell-culture based HBV replication system, we have observed that HBV replication in HepG2 cells results in a cellular morphological phenomenon similar to that activated Rac1, compared to activated Cdc42. Using atomic force microscopy (AFM) and immunostaining, we have shown the similarity in morphology between HBV replicating cells and those expressing activated Rac1. This is particularly evident at the periphery of the cells, displaying membrane rufflings and lamellipodia like structures. Furthermore, we have shown that activated Rac1 results in increased viral replication in conjunction with upstream Cdc42 activation. Using a p21-GTPase binding assay, we have shown that HBV replicating cells displayed a specific activation of transfected WT Rac1 compared to Cdc42. We therefore hypothesize that HBV activation of Rac1 may be the key to the downstream phosphorylation of ERK1/2 and AKT, previously shown to be activated by HBV replication. Using mutants of the GTPases, we have shown that HBV may activate Rac1 independently from its known upstream Cdc42 activation. We propose an activation mechanism by HBx interaction with a RhoGEF ( $\beta$ PIX) through a SH3 binding motif. Such an activation maintains activated Rac1 at its GTP-bound state. Taken together, we have shown for the first time the interaction of HBV with the Rho GTPase,
affecting cell morphology through the Rac1 activation pathway. In addition, HBV may make use of an activated Rac1 signaling pathway for increased replication and resultant metastatic effects. Our results strongly suggest that increased HBV replication is tightly coupled to Rac1 activation and a sustained Rac1 activation may lead to increased viral replication through the interaction with $\beta$ PIX.

As summarized in Figure 5.11, HBV may activate Rac1 independently of the typical upstream Cdc42 activation leading to the morphological changes observed in our AFM and immunostaining analyses. This activation of Rac1 by HBV replication could be in part due to interaction of HBx , a HBV viral protein, interaction with $\beta$ PIX leading to the localization and activation of Rac1 to the Rac1-GTP form. The downstream MAPK and PI-3K activation of Rac1 will result in the phosphorylation of ERK1/2 and AKT respectively. Hence, previous studies of HBV causing ERK1/2 and AKT phosphorylation (Panteva et. al., 2003) and Rac1 activation causing the same effects might be due to this key interaction. Besides the potential of causing hepatocellular carcinoma, the maintained Rac1 activation might result in decrease cell adhesion, increased migration, typical of metastatic liver cancer cells as described above. It was also recently reported that the Rho GTPase might play a role in conferring drug resistance in patients undergoing chemotherapy for hepatocellular carcinoma besides causing cell proliferation (Sterpetti et. al., 2006). Therefore, it would be of good interest to further understand the interaction between HBV and these Rho GTPases which might potentially serve important role for drug design intervention.


Downstream disease outcome

Figure 5.11: A schematic diagram depicting the potential of HBV replication interfering with Rac1 activation, potentially through HBx interaction with $\beta$ PIX, leading to morphological changes as well as activation of signaling pathways MAPK and PI3K/AKT through phosphorylation.

Acknowledgement: Atomic Force Microscopy (AFM) work was performed in conjunction with Dr Fang Ning (SCBE, NTU). Cells were transfected and fixed, prior to analysis by her.

## Chapter 6 <br> Conclusion

## Conclusion

Hepatitis B virus (HBV) infection remains a global threat, affecting more then 350 million people worldwide, most of whom as unknowing carriers of the liver-disease causing virus. HBV is also a known causative factor for various serious liver diseases such as liver cirrhosis as well as hepatocellular carcinoma. Liver cancer remains as the top killer of many individuals of the world due to limited treatment options. Henceforth, it is important to understand how hepatitis viruses interact with the host cells and cause deregulation of normal cellular pathways leading to cancer development.

A recombinant vaccine had been developed against Hepatitis B virus in the early 1980s. Despite its universal success in preventing HBV infection and transmission, it has no therapeutic effects on those who have already contracted the virus. It is therefore a big challenge to develop effective antivirals for the benefits of the chronic carriers. Although nucleoside analogues have been used as antivirals for HBV carriers, the issue of emerging mutant viruses which are resistant to these antivirals remains to be addressed. Part of the reason for the emergence of antiviral-resistance mutants is due to the narrow target if the antivirals, which is the catalytic site of the HBV polymerase.

Development of new antivirals with a wider range of actions would therefore be needed for more effective therapy. A better understanding of the HBV-host interaction should provide useful information on the design of these new antivirals, and also on the underlying mechanism of disease progression associated with HBV infection.

HBV genome encodes for various viral proteins such as surface, polymerase, core and X. The surface and core serves as important "shells" for packaging of the viral genome on successful infection while polymerase takes part in the reverse transcription of the pregenome. HBx , the smallest non-structural viral protein encoded by the HBV genome have been a hot area of research due to its potential carcinogenic effects on transfected cells. Although HBx have been found to be important in maintaining HBV viral replication, as well as its activation of various signaling transduction cascades, data involving direct interactions have been few.

Global differences between the different genotypes of HBx transiently transfected into cells have been established in this thesis. There appear to be some proteins which are attenuated similarly in both genotypes B and C compared to control. On the other hand, differences of proteome expression albeit minor can be observed among the genotypes. Further work needs to be done to characterize the other proteins of difference. The difference in proteome profiles could be useful for biomarker discovery. This can be coupled with the 2 DE analysis on cells transfected with the replicative genome of various genotypes of HBV. Recent developments in the field of liquid chromatography mass spectrometry should also help to elucidate more differential protein expression and overcome the limitation of 2DE analysis (gel to gel variation, sensitivity of detection). Recently, the importance of harnessing such proteomics technique for understanding early HCC development has been discussed (Feng et. al., 2006).

The cellular signaling pathways links and controls many critical cellular process keeping the cellular environment in constant homeostasis. Therefore, it is not surprising that external pathogens such as bacteria or viruses develop strategies or mutate to make use of these pathways for sustaining their replication or defense against host immune mechanism. However, through this process, cellular balance might be augmented and hence result in disease progression. Viruses like HIV and HCV have been previously shown to interact with signaling modules through their non-structural viral proteins, Nef and NS5A respectively. Similar to HBx of HBV, these proteins play unknown role but have been implicated in their respective viral replication. The Nef and NS5A protein interacts with SH3 domain of various cellular proteins through their proline-rich motifs (Saksela et. al., 1995, Tan et. al., 1999). Therefore, it could be likely that HBx acts through similar mechanisms of interaction with cellular proteins.

We report the presence of a variable region between the HBx of genotypes $\mathrm{A}, \mathrm{B}$ and C. This particular variable region consists of the proline-rich segment spanning between amino acids $31-43$. Although previous reports have mentioned a proline / serine rich domain of HBx (Sirma et. al., 1999), few reports have studied the mechanistic aspects of this domain. Our data reveals for the first time PXXP motifs in genotype B HBx , and non-canonical proline-rich motifs in other genotypes of HBx. These prolinerich motifs may play important role in viral attenuation of cellular homeostasis via their binding to the Src Homology 3 (SH3) domain, a common signaling modular domain to many important proteins governing the cytoskeletal maintenance as well as signaling cascades. Hence, we hypothesize that HBx might be important in direct protein-protein
interactions with these cellular proteins through the proline-rich motif and hence harness cellular machinery for viral replication. In the process, HBx might attenuate normal pathways or augment normal cytoskeletal architecture hence, potentiating cells towards hepatocarcinogenesis.

From our array screening, HBx has been found to interact with various proteins including c-Src, Vinexin $\beta$, Cortactin, $\beta$ PIX and others. We have further characterized some of this interactions. Vinexin $\beta$ has been chosen in this project due to its importance in focal adhesion complex formation. Indeed, HepG2 cells transfected with HBV replicative genome show a constant decrease in initial adhesion kinetics and this particular adhesion delay is amplified by transfection of HBx alone. Vinculin, a binding protein of Vinexin $\beta$ (Takahashi et. al., 2005), is shown to be recruited to the focal adhesion complexes at a slower rate in HBV replicating liver cells. Interestingly, HBx mutant with proline - alanine mutations in the proline-rich region, restores the normal adhesion to a level of that of empty vector transfected HepG2 cells. Our data show that HBx potentially plays a role in deregulation of initial cell adhesion through the prolinerich region. Although the effects of focal adhesion deregulation we have observed are only upon initial cellular seeding, it is possible that prolonged attenuation of this critical protein of focal adhesion formation results in alteration in the overall cellular behaviour, and therefore may be associated with carcinogenesis. Another interacting protein of HBx that plays an important role in cytoskeleton, Cortactin has also been identified in our study. It is a cortical actin-binding protein and has been previously reported to be made
use of by various pathogens to transport themselves into the cells (Selbach and Backert, 2005).

Although an activation of the Src kinase by HBx, possibly through the wellestablished global initiation by calcium signaling (Klein and Schneider, 1997), has been reported, no direct interaction between the two protein have been identified. Through the array screening which reveals the interaction between $\mathrm{c}-\mathrm{Src}$ and HBx , we postulate that there might also be a direct interaction between HBx and Src through this proline-rich interacting motif, allowing direct activation of Src. Further work is being carried out on this aspect.

We have observed perturbations at the periphery of HBV replicating cells reminiscent of those effected by activated Rho GTPase, Rac1. Making use of the various Rho GTPases mutants, we have found that HBV causes Rac1 activation independent of upstream Cdc42 activation. In addition, this Rac1 activation is shown to be able to upregulate HBV viral replication. We have also found that HBx , which has effected cytoskeleton changes as observed previously, directly impacts on these membrane perturbations. Indeed, cells transfected with HBx show membrane rufflings and lamellipodia as observed in HBV replicating cells. This membrane rufflings are not observed in untransfected cells. We postulate that HBx may cause Rac1 activation through interaction with a protein $\beta$ PIX through the proline-rich motif and hence help in viral replication. Rac1 V12 mutant has been shown to display transformed phenotype, including the increased ability to grow on soft agar as well as a lower dependency on
serum for their growth (Boivin et. al., 1996). Hence, a sustained activation of Rac1 by HBV replication may have transforming effects on the hepatocytes.

HBx is a multifunctional protein which interacts promiscuously to many cellular proteins. Hence, its not surprising that HBx interferes with the cytoskeletal network, probably in an attempt to harness the machinery for viral proteins transport or maintenance of viral replication. During this process, prolonged activation may result in carcinogenic effects observed by HBx as previously reported. More studies conducted on this proline-rich domain should provide a better understanding of HBx effects in liver cells and new clues on drug design. It has already been shown that artificial SH3 domain ligands for the Nef proline-rich have resulted in decreased HIV viral replication (Hiipakka et. al., 2001) upon application. Similar mechanism of therapy may also work on HBV if a specific SH3 domain can be found for each HBx.

Besides Vinexin $\beta$, another important interacting protein, Cortactin is found by the SH3 domain array (Figure 5.8 panels B and C). Cortactin is named as it is a cortical protein with actin binding capability. They play an important role in the regulation of the cytoskeleton. The overall mechanisms of activation remain unclear (Lua and Low, 2005) despite reports on phosphorylation of residues on cortactin from several models. It plays important role for activation of the Arp2/3 complex required for cytoskeletal adhesion (Bershadsky, 2004). Interestingly, it has also been found through screening of oligonucleotides array, that the metastases and increased in motility of HCC cells are due to an overexpression of cortactin (Chuma et. al., 2004). Of interest, pathogens such as

Listeria, Shigella and Vaccinia have been known to make use of cortactin, to augment the cytoskeleton for their pathogenic transport (Selbach and Backert, 2005). Besides recruitment by these pathogens, cortactin recruitment to the sites for actin polymerization has been found to involve Rac1 (Weed et. al., 1998). HBx could therefore be an interacting partner of Cortactin.

To this end, we have conducted preliminary investigations using various biochemical pulldown assays to validate the binding of Cortactin, both bacterially expressed as well endogenous cortactin (from mammalian cell culture) to bind the proline-rich HBx construct. Cortactin has been expressed as a GST-fusion in pGEX-5X-1 as observed in Appendix Figure 9.8 (lane 2). The fusion protein was digested with Factor Xa (GE healthcare) to remove the GST moiety for interaction purpose and ran in lane 1. As observed in Figure 9.8, cortactin (after removal of the GST moiety by Factor Xa) interacted with GST-PXXP-HIS (lane 6) and with GST (lane 4). Little binding was observed in GST-AXXA-HIS at lane 8. We further went on to confirm the interaction with endogenous Cortactin (from mammalian cells). As observed in Appendix Figure 9.9, total protein lysates were probed for cortactin using rabbit polyclonal anti-cortactin antibodies (Santa Cruz Biotech). As observed, both Huh7 and HEK293T cells showed positive Western for cortactin in the total protein lysates. Therefore, we subjected the total protein to interaction using GST pulldown with the same panel of protein as Figure 9.8. As observed in Figure 9.10 (B), similar binding trend was observed as in Figure 9.8. The loading consistency of the bound proteins (GST, GST-PXXP-HIS and GST-AXXAHIS) can be observed by Coomassie blue staining in Figure 9.10 (A). Also, the
interaction protein subjected for interaction was constant as observed in both the Coomassie and Western analysis of cortactin in Figures 9.10 (C) and (D). However, more work needs to be performed to understand how HBx interaction with Cortactin might play important role in HCC development.

Taken together, the differences in HBV genotypes nucleotide sequences may be linked to differential disease outcome. This is likely attributed to the downstream differential transcription of viral proteins. In particular, the small and relatively conserved X protein has been shown to be highly variable in the proline-serine rich region. Using constructs of the different genotypes of HBx, we have shown the differential protein expression profiles effected by their individual transfection compared to untransfected cells. The proline-rich motifs identified in HBx may play a role in binding of important SH3 domain-containing cellular proteins. Significantly, HBx binds to Vinexin $\beta$ through this proline-rich motif and results in a delay of focal adhesion formation leading to an overall decrease in adhesion kinetics in HBV replicating and HBx transfected cells. This effect of slower adhesion can be rescued to normal level when the prolines are replaced with alanines. In addition to the cell adhesion process, we have also demonstrated a link between HBx and the overall HBV replication by the morphological changes in the cells either producing HBx or sustaining HBV replication. The membrane perturbations in HBV replicating cells are similar to HBx transfected cells. Concurrently, HBV replication is found to cause Rac1 activation, and appears to occur downstream of the usual Cdc42 activation. The activation of Rac1 by HBV has been supported by the interaction between HBx and $\beta$ PIX, a known activator of Rac1. The overall effects of

Rac1 activation by HBV replication has been found to be a sustainable HBV replication (Appendix Figure 9.7).

Our investigation has therefore provided molecular evidence on the interference of HBV replication with cellular signaling pathway. The identification and characterization of SH 3 -binding motif in the promiscuous HBx protein have shed new lights on the mechanism of and action in HBV-host interaction, and the direct involvement of HBx in HBV replication. More importantly, our studies have shown a deregulation of the focal adhesion complex formation as well as perturbation of the Rho GTPases as a result of HBV replication. These impact directly on overall cellular behavior with a decrease in cell adhesion and an increase in cell migration. This in turn may have important implications in metastases of carcinoma cells.

## Chapter 7 Future Directions

## Future Directions

One of the important future works would be to further understand and find early biomarkers of HBV-related HCC development. A primary human hepatocytes susceptible to HBV viral infection recently established in our lab should yield better results in terms of relevance. Coupling of proteomics analyses with recently developed Liquid Chromatography Mass Spectrometry may lead to the detection of non-abundant proteins with biomarker property.

The interesting 102-bp deletion described in Chapter 3 warrants further clinical investigation to understand its potential effect on chronic HBV carriers. Particularly, serum samples could be screened for such deletions and correlated with patient's clinical presentations to obtain significant understanding of its role.

Various interacting proteins of the cytoskeleton and cellular signaling cascades have been found to potentially interact with HBx proline-rich region. Further work would focus on these differential binding related to the different genotypes. This should allow a better understanding of how HBx of different genotypes affect cells via specific SH 3 binding. Another focus would be on detailed analysis of Cortactin in the context of HBx interaction. This is interesting as either an upregulation of its synthesis or a deregulation of its normal functioning in maintaining the cytoskeleton may have deleterious effects.

Proteins of the signaling cascades such as Src kinases might potentially be directly or indirectly activated by binding of HBx to them directly or via their adaptor
proteins. It would be interesting to study exactly which protein HBx might bind to so as to elicit their effects. As such, it would allow for understanding of many of the previous unanswered questions as to how HBV replication can cause downstream activation of signaling molecules.

Transgenic mice carrying either wild type HBx or HBx mutated in the SH 3 binding domain (prolines to alanines) should provide useful insights on the biological relevance of our study. In particular, the rate of nodular formation after the induction of tumor formation through application of carcinogens can be compared in HBx and HBx mutant.

Validation of our findings in transgenic animals may lead to development of new therapeutic agents. One example is to use SH3 domains that are able to compete specifically for viral proteins such that the normal cellular signaling is protected from interference by viral replication. It is to be hoped that developments of such new antivirals with wider range of action should improve the overall therapeutic efficiency for HBV carrier.

## Chapter 8 <br> References

## References


#### Abstract

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## Chapter 9 Appendix

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 Figure 9．1：Alignment of NCBI database sequences for genotypes B and C．Note that all ORFs are marked with an arrow（ $\boldsymbol{P}$ ） and boxed regions are conserved sites for primer design．Note the higher incidence of G1896A in genotype $B$ sequences compared to $\mathbf{C}$（mutation boxed in red）．








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Figure 9.2: HBV replicative genome Cloning Strategy. It consists essentially of two digestion and re-ligation processes with the HBV being PCR into 2 overlapping fragments 1 and 2. Fragment carries a unique restriction site for fragment 2 as designated

|  | 1 | 100 |
| :---: | :---: | :---: |
| B_101Del | (1) | --GGGAGGTTGGTCTTCCAAACCTCGAAAAGGCATGGGGACAAATCTTTCTGTCCCCAATCCCCTGGGATTCTTCCCCGATCATCAGTTGGACCCTGCAT |
| B_Surface | (1) | ATGGGAGGTTGGTCCTCCAAACCTCGAAAAGGCATGGGGACAAATCTTTCTGTCCCCAATCCCCTGGGATTCTTCCCCGACCATCAGTTGGACCCTGCAT |
|  |  | 101200 |
| B_101Del | (99) | TCAAAGCCAACTCAGAAAATCCAGATTGGGACCTCAACCCGCACAAGGACAACTGGCCGGACGCCAACAAGGTGGGAGTGGGAGCATTCGGGCCAGGGTT |
| B_Surface | (101) | TCAAAGCCAACTCAGAAAATCCAGATTGGGACTTCAACCCGCACAAGGACAACTGGCCGGACGCCAACCAGGTGGGAGTGGGAGCATTCGGGCCAGGGTT |
|  |  | 201300 |
| B_101Del | (199) | CATTCCACCCCACGGGGGACTGTTGGGGTGGAGCCCTCAGGCTCAGGGCATACACACAACTGTGCCAGCAGCTCCTCCTCCTGCCTCCACCAATCGGCTG |
| B_Surface | (201) | CATTCCGCCCCATGGGGGACTGTTGGGATGGAGCCCTCAGGCTCAGGGCATACTTACAACTGTGCCAGCAGCTCCTCCTCCTGCCTCCACCAATCGGCAG |
|  |  | 301 ( 400 |
| B_101Del | (299) |  |
| B_Surface | (301) | TCAGGAAGGCAGCCTACTCCCTTATCTCCACCTCTAAGGGAGACTCATCCTCAGGCCATGCAGTGGAACTCCACCACTTTCCACCAAACTCTTCAAGATC |
|  |  | 401 ( 500 |
| B_101Del | (300) | ---GAGTCAGGGCCCTGTACTTTCCTGCTGGTGGCTCCAGTTCAGGAACAGTGAGCCCTGCTCAGAATACTGTCTCTGCCATATCGTCAATCTTATCGAA |
| B_Surface | (401) | CCAAAGTCAGGGCCCTGTACTTTCCTGCTGGTGGCTCCAGTTCAGGAACAGTGAGCCCTGCTCAGAATACTGTCTCAGCCATATCGTCAATCTTATCGAA |
|  |  | 501 600 |
| B_101Del | (397) | GACTGGGGACCCTGTACCGAACATGGAGAACATCGCATCAGGACTCCCAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTCGTTGACAAAAATCCTC |
| B_Surface | (501) | GACTGGGGACCCTGTACCGAACATGGAGAACATCGCATCAGGACTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAAAATCCTC |
|  |  | 601700 |
| B_101Del | (497) | ACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAGTTTTCTAGGGGGAACACCCGTGTGTCTTGGCCAAAATTCGCAGTCCCAAATCTCCAGTC |
| B_Surface | (601) | ACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGGACACCCGTGTGTCTTGGCCAAAATTCGCAGTCCCAAATCTCCAGTC |

Figure 9.3: 102bp deletion mutant in cloned replicative HBV genome, genotype B within the PreS1 region to PreS2 region of Surface Antigen. This in frame deletion mutant is unique and has not been studied before. Orange highlight and blue highlights indicates the start codon of PreS1 and Surface ORF. PreS2 start codon is further upstream from the above sequences.


Figure 9.4: Comparison of the database amino acid sequences of the HBV viral protein, HBx. Above sequences were obtained by in silico translation of the HBX ORF using database sequences of HBV genomes as shown in Figure 12. (a) shows the difference in serine-proline rich region of the two genotypes and (b) shows the HRN and RQV conserved in between genotype $B$ and C .


Figure 9.5: 2DE of proteins prepared from untransfected Chang Liver cells culture media. Potential as use for screening secretable biomarkers by HBV-transfected cell culture.

## Vinexin $\alpha$



## Vinexin $\beta$



Figure 9.6: Structural comparison of Vinexin. Vinexin $\alpha$ and Vinexin $\beta$ defers only by the N-terminal portion. Both retain the 3 Sre Homology domain (SH3) namely demarcated by 1, 2 and 3 respectively. In particular, HBx interacted with SH3 \#3 (marked with red asterix) and not \#1. Vinexin $\alpha$ possess an additional $N$-terminal Sorbin homology domain (SOHO). (Adapted from: Kioka et. al., 1999)


Figure 9.7: Cdc42 acts upstream of Rac1. As seen above, activation of Rac1 occurs downstream of Cdc42 and is mediated through PIX. HBx might interact (as indicated with red arrow) with PIX and resulting downstream activation. These might feedback to increasing HBV replication. Also, the interaction might have deleterious effect if there is no negative control potentially leading to carcinogenesis and metastases (Green arrows).
(Adapted from: Obermeier et. al., 1998)


Figure 9.8:
GST-pulldown analysis of GST-digested Cortactin. Cortactin was expressed as a GST fusion of $\sim 107 \mathrm{kDa}$ as shown in Lane 2. Lane 1 depicts the factor Xa (GE healthcare) digested band of Cortactin ( $80-85 \mathrm{kDa}$ ) from the fusion protein. $M$ represents RPN 756 (GE healthcare), a protein molecular weight marker (the various molecular weights are stated on the left). Lanes 4, 6 and 8 represents GST, GST-PXXP-HIS and GST-AXXA-HIS of genotype A respectively after pulldown analysis. Lanes 3,5 and 7 represent the flow through from the pulldown assay. As indicated by the red arrow, it seems that GST-PXXP-HIS (Lane 6) showed a pulldown of digested Cortactin compared to nothing on GST (lane 4) and lesser amounts on GST-AXXA-HIS (Lane 8)
(A)

(B)


Figure 9.9: Western analysis for endogenous Cortactin in mammalian cells. Total protein lysates from (1) HuH7 as well (2) 293T cells were separated on (A) $\mathbf{1 2 \%}$ SDS-PAGE and transferred onto nitrocellulose membrane. Polyclonal Rabbit antiCortactin (Santa Cruz) was used in the concentration of 1:5000 with $3 \%$ non-fat milk/ 1X PBS. Goat anti-Rabbit secondary antibodies, conjugated with HRP (Pierce) was used in the concentration of $1: 10,000$ with $3 \%$ non-fat milk/ 1X PBS. (B) shows the developed film with substrate and luminol from Pierce WestPico chemiluminescence.


Figure 9.10: GST pulldown analysis using GST (-ve control), GST-PXXP-His and GST-AXXA-His expressed proteins to pull down potential interacting proteins from Hek293T cell lysates. Rabbit polyclonal anti-cortactin antibodies were used to detect for the endogenous cortactin after GST pulldown. Figures A and B shows the Pulldown results using proteins eluted from the beads. Lanes 1,2 and 3 represents GST, GST-PXXP-His and GST-AXXA-His respectively. Figures C and D depicts the un-interacted cortactin within the endogenous lysates after the interaction.


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