BIOLOGICAL FUNCTIONS OF INTRACELLULAR HEPATITIS B e ANTIGEN

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DEDICATION

I am extremely fortunate to have been raised by the two most loving and inspiring parents- Mr. Bibhash Kanti Mitra and Mrs. Sudipta Mitra. They have always influenced me and shaped me into the person I am today. I shall forever be indebted to them for the innumerable sacrifices that they have made to provide me with a better education and a life of opportunities. My maternal grandparents- (Late) Sunil Baran Majumder and (Late) Prova Majumder- have been my most sincere cheerleaders since I have known, and it breaks my heart to not have them here today to celebrate the moment they have always wanted to see. My family has been my pillar of mental strength from 13000 miles away whenever I needed them, and I feel immensely blessed to have been born in this family. As a small token of my gratitude, I dedicate this thesis to my family.

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BIOLOGICAL FUNCTIONS OF INTRACELLULAR HEPATITIS B e ANTIGEN

The function(s) of the intracellular form of HBeAg, previously reported as the preCore protein intermediate (p22) without the N-terminal signal peptide, remains elusive. Here, we propose to elucidate the translocation of p22 during its formation from endoplasmic reticulum (ER) to cytosol, how it differs from core in its inability to form a capsid and the biological functions of cytoplasmic and nuclear p22. Firstly, we have identified that a portion of p22, after the cleavage of its signal peptide in ER, is released back into the cytosol through an ERAD-independent mechanism, as neither wildtype nor dominant-negative p97 affected the ER-to-cytosol translocation of p22 or ER-Golgi secretion of HBeAg. Secondly, despite sharing the same sequence with core protein except for the extended 10 amino acid precore region at the N-terminus, we observed that p22 wildtype and C-7Q mutant are unable to form a capsid. Thirdly, we report that p22 but not the secreted HBeAg significantly reduced interferon stimulated response element (ISRE) activity and expression of interferon stimulated genes (ISGs) upon interferon-alpha (IFN-α) stimulation. Furthermore, in line with this, RNA-seq analysis of ISG induction profile from IFN-α treated patients showed that HBeAg(+) patients exhibited reduced and weak antiviral ISG upregulations compared to HBeAg(-) patients. Further, mechanistic study indicated that while p22 did not alter the total STAT1 or p-STAT1 levels in IFN-α treated cells, it blocked the nuclear translocation of p-STAT1 by interacting with karyopherin α1, indicating that the cytoplasmic p22 may impede JAK-STAT signaling to help the virus evade host innate immune response and cause resistance to IFN therapy in patients. Additionally, nuclear p22 and nuclear core were found to interact with the promoter regions

(ISRE – containing) of ISGs, suggesting a new mechanism of inhibition of ISG expression upon stimulation. Finally, we found that the nuclear p22 can bind to cccDNA minichromosome and affects cccDNA maintenance and/or transcription. Thus, our results indicate that there is a novel ER sorting mechanism for the distribution of the intracellular and secretory HBeAg, and the intracellular HBeAg may contribute to HBV persistence by interfering with IFN-α elicited JAK-STAT signaling and regulating cccDNA metabolism.

Haitao Guo, Ph.D., Chair

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LIST OF ABBREVIATIONS

HBV	Hepatitis B Virus
HDV	Hepatitis Delta Virus
rcDNA	relaxed Circular DNA
cccDNA	covalently closed circular DNA
pgRNA	pregenomic RNA
pcRNA	precore RNA
ORF	open reading frame
S	HBV surface protein
C	HBV core protein
X	HBV X protein
P	HBV polymerase protein
Pol	HBV polymerase protein
RT	Reverse Transcriptase
DR	Direct Repeats
En	Enhancer
GRE	Glucocorticoid-responsive element
HSPG	Heparan sulfate proteoglycan
AGL	Antigenic Loop
GPC5	Glypican-5
NTCP	Sodium Taurocholate Cotransporting Polypeptide
ERAD	Endoplasmic Reticulum Associated Degradation
SRP	Signal Recognition Complex

VCP	Valosin-containing Protein
UPS	Ubiquitin–proteasome system
HBeAg	Hepatitis B e Antigen
HBcAg	Hepatitis B core Antigen
HBsAg	Hepatitis B surface Antigen
HBxAg	Hepatitis B X Antigen
Anti-HBe	HBe Antibodies
Anti-HBc	Core Antibodies
Anti-HBs	Surface Antibodies
IFN-α/β/γ	Interferon-alpha/beta/gamma
Peg-IFNα	Pegylated Interferon-alpha
JAK	Janus Kinases
STAT	Signal Transducer and Activator of Transcription
ISG	Interferon Stimulated Genes
MxA	Myxovirus resistance protein 1
OAS2	2'-5'-Oligoadenylate Synthase 2
IRF	Interferon Regulatory Factor
pSTAT	phosphorylated-Signal Transducer and Activator of Transcription
NF-κB	Nuclear Factor-κB
TNF	Tumor Necrosis Factor
MDA-5	Melanoma Differentiation Associated Gene-5
RIG-I	Retinoic acid-inducible gene I
TLR	Toll-like Receptor

RLR	RIG- I -like receptors
NK	Natural Killer cells
NKT	Natural Killer T cells
DC	Dendritic Cells
Tregs	Regulatory Cells
MyD88	Myeloid Differentiation Factor-88
TRIP	TIR-domain-containing adaptor-inducing IFN-β
IRAK	Interleukin-1 Receptor-Associated Kinase
IPS-1	IFN Promoter Stimulator 1
MAVS	Mitochindrial Antiviral-Signaling Protein
VISA	Virus-induced Signaling Adaptor
IL	Interleukin
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
AFP	Alpha-fetoprotein
CHB	Chronic Hepatitis B
HCC	Hepatocellular Carcinoma
Ig	Immunoglobulin
CpAM	Core protein Allosteric Modulators
HAP	Heteroaryldihydropyrimidine
SBA	Sulfamoylbenzamide
PPA	Phenylpropenamides
DAA	Direct Antiviral Agent

HTA	Host Targeting Agents
H3	Histone 3
H3K27Ac	Histone 3 Lysine 27 Acetylation
Tet	Tetracycline
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
PHH	Primary Human Hepatocytes
HepG2	Human Liver Hepatocellular Carcinoma cell
293T	Human Embryonic Kidney 293, SV40 large T Antigen
WT	Wild Type
aa	Amino Acid
Cys	Cysteine
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
ER	Endoplasmic Reticulum
BFA	Brefeldin A
VCP	Valosin-containing Protein
SPase	Signal Peptidase
CTD	C-terminal Domain
PCR	Polymerase Chain Reaction
VSV	Vesiculovirus

CHAPTER 1

INTRODUCTION

1.1 HEPATITIS B

Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV). It is one of the major global health concerns as the chronically infected patients are at high risk of death from cirrhosis and/or liver cancer(1, 2). Approximately 250 million people worldwide are chronically infected with HBV and more than 780,000 people die every year due to hepatitis B-associated complications, including cirrhosis and liver cancer (3).

1.1.1 ACUTE HEPATITIS

About two-thirds of patients with acute HBV infection have a mild, asymptomatic and subclinical illness that usually goes undetected(4). Approximately one-third of adults with acute HBV infection develop clinical symptoms and signs of hepatitis, which range from mild constitutional symptoms of fatigue and nausea, to more marked symptoms and jaundice, and rarely to acute liver failure. The clinical incubation period of acute hepatitis B averages 2–3 months and can range from 1–6 months after exposure, the length of the incubation period correlating, to some extent, with the level of virus exposure(5). The incubation period is followed by a short preicteric or prodromal period of constitutional symptoms such as fever, fatigue, anorexia, nausea, and body aches. During this phase, serum ALT levels rise and high levels of HBsAg and HBV DNA are detectable. The preicteric phase lasts a few days to as long as a week and is followed by onset of jaundice or dark urine. The icteric phase of hepatitis B lasts for a variable period averaging 1–2

weeks, during which viral levels decrease. In convalescence, jaundice resolves but constitutional symptoms may last for weeks or even months. During this phase, HBsAg is cleared followed by the disappearance of detectable HBV DNA from serum. Acute liver failure occurs in approximately 1% of patients with acute hepatitis B and jaundice(6). The onset of fulminant hepatitis is typically marked by the sudden appearance of fever, abdominal pain, vomiting, and jaundice, followed by disorientation, confusion, and coma. HBsAg and HBV DNA levels generally fall rapidly as liver failure develops, and some patients are HBsAg-negative by the time of onset of hepatic coma. Patients with acute liver failure due to hepatitis B require careful management and monitoring and should be referred rapidly to a tertiary medical center with the availability of liver transplantation (7).

1.1.2 CHRONIC HEPATITIS

Chronic hepatitis B has a variable and dynamic course. Early during infection, HBeAg, HBsAg, and HBV DNA are usually present in high titers, and there are mild to moderate elevations in serum aminotransferase levels. With time, however, the disease activity can resolve either with persistence of high levels of HBeAg and HBV DNA (the "immune tolerance phase") or with loss of HBeAg and fall of HBV DNA to low or undetectable levels ("inactive carrier state")(7). Other patients continue to have chronic hepatitis B, although some lose HBeAg and develop anti-HBe (HBeAg-negative chronic hepatitis B). The course and natural history of hepatitis B are discussed in detail elsewhere in these proceedings(8). The overall prognosis of patients with chronic hepatitis is directly related to the severity of disease. For those with severe chronic hepatitis and cirrhosis, the 5-year survival rate is about 50% (9-11). Among patients with evidence of chronic hepatitis

(elevated ALT and inflammation and/or fibrosis on liver biopsy), many are asymptomatic or have nonspecific symptoms, such as fatigue and mild right upper quadrant discomfort. Patients with more severe disease or cirrhosis may have significant constitutional symptoms, jaundice, and peripheral stigmata of end-stage liver disease including spider angiomata, palmar erythema, splenomegaly, gynecomastia, and fetor hepaticus. Ascites, peripheral edema, encephalopathy, and gastrointestinal bleeding are seen in patients with more advanced cirrhosis. ALT and AST are often elevated but may not correlate well with severity of liver disease. Bilirubin, prothrombin time, and albumin often become abnormal with progressive disease. Decreasing platelet count is often a poor prognostic sign. Patients with chronic hepatitis may develop acute exacerbations with markedly elevated serum ALT. This scenario is more frequently described in those with HBeAg-negative chronic hepatitis B. To distinguish between acute hepatitis B and chronic hepatitis B with a flare, antiHBc IgM is a useful marker. However anti-HBc of the IgM class can be detected occasionally in patients with chronic hepatitis B with exacerbation. Alpha-fetoprotein (AFP), used as a marker for HCC, is often elevated in parallel with ALT during acute exacerbation(12). However, it is unlikely to exceed 400 ng/mL. In patients with AFP much greater than this level, development of HCC should be suspected. Chronic HBV infection progresses to cirrhosis in up to 40% of untreated patients and an estimated one-third of persons with chronic HBV infection will ultimately develop a long-term consequence of the disease, such as cirrhosis, end-stage liver disease, or HCC. The determinants of outcome of chronic hepatitis B appear to be both viral (HBV DNA levels, HBV genotype, and some HBV mutation patterns) and host-specific (age, gender, genetic background, and immune status). Chronic HBV infection accounts for at least 50% of HCC cases (13, 14)

and primary liver cancer (approximately 75%-90% of cases are HCC) is the second most common cause of death from cancer in the world(15). Among patients with HBV infection, HCC can develop in the absence of cirrhosis (approximately 10% of cases in a large Veteran's Affairs cohort of 8539 patients)(16); however, HCC is typically preceded by cirrhosis (70%-90% of patients)(17).

1.1.3 PRESENTATION

Individuals with chronic HBV infection are typically asymptomatic and are diagnosed during routine health maintenance or screening (e.g., blood donation or an evaluation for an elevated level of liver enzymes). Among adults with acute HBV infection, only 5% to 10% will progress to chronic HBV infection. Only one-third of adults develop symptoms (e.g., fever, fatigue, malaise, abdominal pain, and jaundice) during an acute HBV infection. The remainder have subclinical or asymptomatic illness that may be undetected(7, 13).

1.1.4 DIAGNOSIS

HBV infection leads to a wide spectrum of liver disease ranging from acute hepatitis (including fulminant hepatic failure) to chronic hepatitis, cirrhosis, and HCC(7). The diagnosis of HBV infection and its associated disease is based on a constellation of clinical, biochemical, histological, and serologic findings. A number of viral antigens and their respective antibodies can be detected in serum after infection with HBV, and proper interpretation of the results is essential for the correct diagnosis of the various clinical forms of HBV infection. Serological markers can be used to diagnose and distinguish

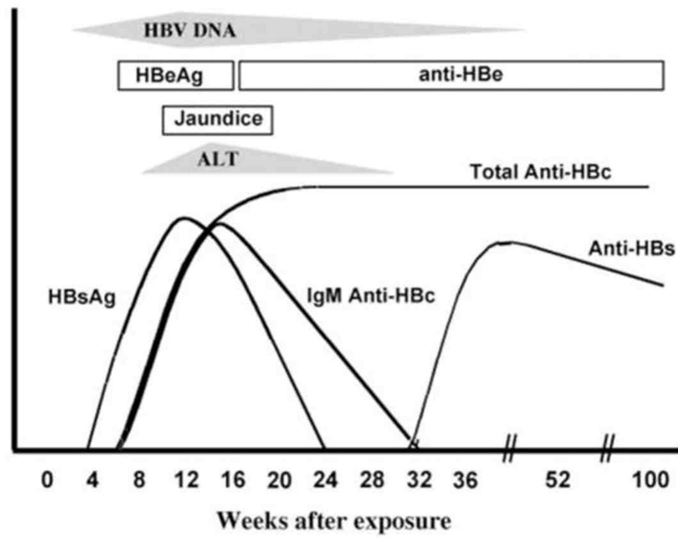
between acute and chronic infections. Commercially available serological tests detect HBV surface antigen (HBsAg), HBV e antigen (HBeAg), HBV surface antibody (anti-HBs), HBV core antibody (anti-HBc), HBV e antibody (anti-HBe), and HBV DNA (13, 18). Chronic HBV infection is defined as detection of HBsAg on 2 occasions measured at least 6 months apart. Individuals at risk for HBV infection (populations with ≥2% prevalence of HBV infection) should be screened with serological tests for the presence of HBsAg, anti-HBs, and anti-HBc, and offered a vaccine if not immune. Among individuals who recover from HBV infection, 80% will develop anti-HBs and all will develop anti-HBc(19). Therefore, testing for anti-HBc is important for identifying individuals who may have been previously infected. Individuals younger than 19 years should be vaccinated. Individuals in high-risk groups who were vaccinated as infants or schoolchildren should still be screened to determine immunity (or lack of) to HBV. Individuals with anti-HBs levels of less than 10 IU/L are considered not immune. The typical course of acute hepatitis B is shown in Picture 1A. HBV DNA followed shortly afterward by HBsAg and HBeAg are the first viral markers detected in serum(20). HBsAg may be detected as early as 1–2 weeks or as late as 11–12 weeks after exposure, and its persistence is a marker of chronicity. HBeAg correlates with the presence of high levels of HBV replication and infectivity(21). Within a few weeks of appearance of viral markers, serum alanine and aspartate aminotransferase (ALT, AST) levels begin to rise and jaundice may appear. HBeAg is usually cleared early, at the peak of clinical illness, whereas HBsAg and HBV DNA usually persist in the serum for the duration of clinical symptoms and are cleared with recovery. Antibodies to the HBV proteins arise in different patterns during acute hepatitis B. Antibody to HBcAg (anti-HBc) generally appears shortly before onset of clinical illness,

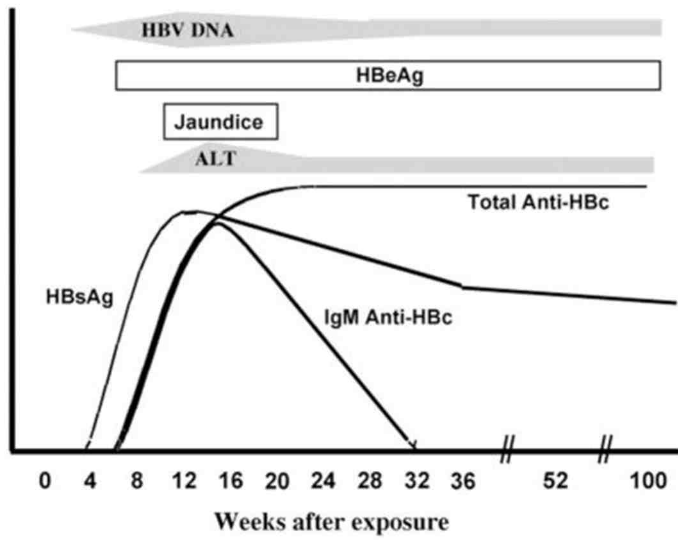
the initial antibody being mostly immunoglobulin M (IgM) class, which then declines in titer as levels of IgG anti-HBc arise. Antibody to HBeAg (anti-HBe) usually appears shortly after clearance of HBeAg, often at the peak of clinical illness. Thus, loss of HBeAg and appearance of anti-HBe is a favorable serological marker during acute hepatitis B, indicating the initiation of recovery. Anti-HBsAg arises late during infection, usually during recovery or convalescence after clearance of HBsAg. Anti-HBs persists after recovery, being the antibody associated with immunity against HBV. However, between 10% and 15% of patients who recover from hepatitis B do not develop detectable anti-HBs and have anti-HBc alone as a marker of previous infection. For this reason, anti-HBc testing is the most reliable means of assessing previous infection with HBV, whereas anti-HBs testing is used to assess immunity and response to HBV vaccine(22).

Patients who develop chronic hepatitis B (Picture 1B) have a similar initial pattern of serological markers with appearance of HBV DNA, HBsAg, HBeAg, and anti-HBc. In these persons, however, viral replication persists and HBsAg, HBeAg, and HBV DNA continue to be detectable in serum, often in high titers. The subsequent course of chronic hepatitis B is quite variable. Most persons remain HBsAg-positive for years if not for life and have some degree of chronic liver injury (chronic hepatitis) that can lead to significant fibrosis and cirrhosis. Persons with chronic HBV infection are also at high risk to ultimately develop HCC.

The diagnosis of acute hepatitis B is reliably made by the finding of IgM anti-HBc in serum, particularly in a patient with HBsAg and signs, symptoms, or laboratory features of acute hepatitis. Nevertheless, in some instances, HBsAg is cleared rapidly from the serum, and IgM anti-HBc is the only marker detectable when the patient presents with

hepatitis. Testing for anti-HBc (total) and anti-HBs are not useful in diagnosis and testing for HBeAg and anti-HBe should be reserved for persons who test positive for HBsAg. The finding of HBsAg without IgM anti-HBc suggests the presence of chronic hepatitis B, but this diagnosis generally also rests upon finding of persistence of HBsAg for at least 6 months(20, 23). HBV DNA testing can also be helpful in the assessment of level of viral replication and possibly helpful in assessing prognosis and need for antiviral therapy. Assays for HBV DNA level have improved substantially over the years(24). The current real-time polymerase chain reaction–based assay has a lower limit of detection of 5–10 HBV DNA copies/mL of blood and can accurately quantify a wide range of levels. With this degree of sensitivity, HBV DNA can be detected early during infection, arising before the appearance of other serological markers, such as HBsAg or anti-HBc. Therefore, testing for HBV DNA has emerged as a primary approach in the diagnosis and management of HBV infection. HBV DNA testing has now become routinely used in blood product screening (nucleic acid testing) (25) and monitoring of patients with HBV during treatment(26). Persistently high levels of HBV DNA following resolution of hepatitis may be indicative of a failure to control the infection and an evolution into chronic infection(7).





Picture 1: The clinical course and serological profiles of (A) Acute and (B) Chronic Hepatitis B

1.2 HEPATITIS B VIRUS

HBV is a non-cytopathic, hepatotropic DNA virus belonging to the *Hepadnaviridae* family(1) causing the potentially life-threatening liver infection, Hepatitis B. The virus is one of the smallest enveloped viruses with a virion diameter of 42 nm. It has some unusual similar features to retroviruses (188, 189). Related viruses are found in woodchucks, ground squirrels, tree squirrels, Peking ducks, and herons. Based on sequence comparison, HBV is classified into eight genotypes, A to H. Each genotype has a distinct geographic distribution.

The infectious virus particle, called Dane particle (virion, 42nm) (Figure 1 A), consists of an outer lipid envelope enclosing an icosahedral nucleocapsid composed of core protein (HBcAg). The outer envelope contains embedded proteins (HBsAg) which are involved in viral binding of, and entry into, susceptible cells. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity similar to retroviruses(27). The genome of HBV is a partially double-stranded circular DNA of about 3.2 kilobase (kb) pairs. The viral polymerase is covalently attached to the 5′ end of the minus strand(28).

Certain pleomorphic forms of HBV also exist, including filamentous (variable lengths with a width of 22 nm) and spherical bodies (diameter of 20 nm) that lack a core. These particles are non- infectious since they lack a capsid packaging the viral genome and are composed of the lipid envelope and HBsAg proteins that forms part of the surface of the virion, and is produced in excess during the life cycle of the virus(28). They are called subviral particles (Figure 1B).

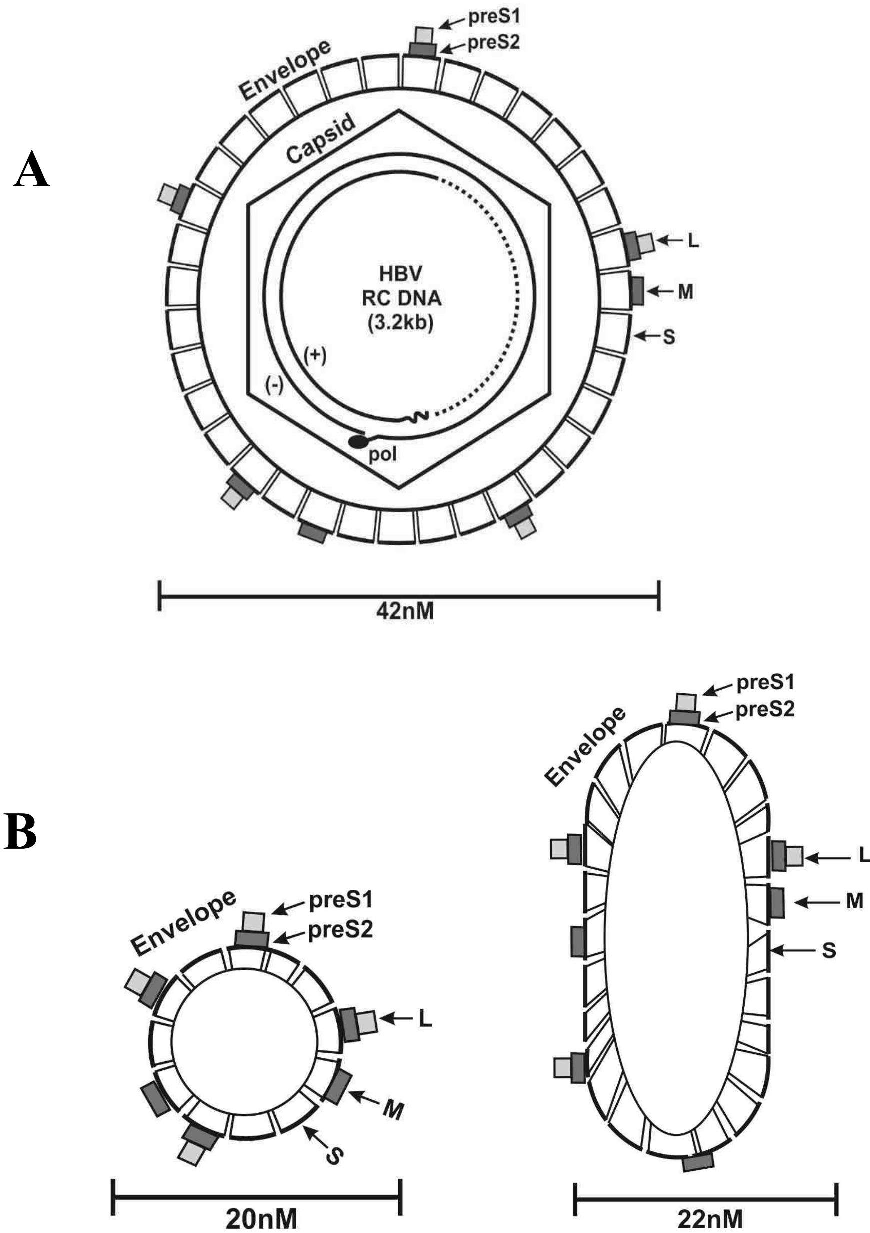


Figure 1: HBV virion, HBV genomes and subviral particles

(A) HBV Dane Particle

The HBV virion is called dane particle which is the infectious particle found within the body of infected patients, This virion has a diameter of 42nm and the outer lipid envelop has high quantity if HBsAgs- L, M, S surface proteins, The envelope encloses a

nucleoclapsid made up of 180 HBV core proteins arranged in icosahedral arrangement. The nucleocapsid encloses the rcDNA HBV genome that has a DNA pol attached to it. (B) Spherical and Filamentous subviral particles. Besides the Dane particle, infected cells also release empty particles that consist of the HBsAg-rich lipid envelop called the subviral particles

1.2.1 HBV GENOME

The genome of HBV is a partially double-stranded circular DNA of about 3.2 kilobase (kb) pairs (Figure 1). The viral polymerase is covalently attached to the 5′ end of the minus strand(29). The viral genome encodes four overlapping open reading frames (ORFs: S, C, P, and X). The S ORF encodes the viral surface envelope proteins, the HBsAg, and can be structurally and functionally divided into the pre-S1, pre-S2, and S regions. The core or C gene has the precore and core regions. Multiple in-frame translation initiation codons are a feature of the S and C genes, which give rise to related but functionally distinct proteins. The C ORF encodes either the viral nucleocapsid HBcAg or hepatitis B e antigen (HBeAg) depending on whether translation is initiated from the core or precore regions, respectively. The core protein has the intrinsic property to self-assemble into a capsid-like structure and contains a highly basic cluster of amino acids at its C-terminus with RNA-binding activity(30). The precore ORF codes for a signal peptide that directs the translation product to the endoplasmic reticulum (ER), where the protein is further processed to form the secreted HBeAg. The function of HBeAg remains largely undefined, although it has been implicated as an immune tolerogen, whose function is to promote persistent infection(31). The polymerase (pol) is a large protein (about 800 amino acids) encoded by the P ORF and is functionally divided into three domains: the terminal protein domain, which is involved in encapsidation and initiation of minus-strand synthesis; the reverse transcriptase (RT) domain, which catalyzes genome synthesis; and the ribonuclease H domain, which degrades pregenomic RNA and facilitates replication. The HBV X ORF encodes a 16.5-kd protein (HBxAg) with multiple functions, including signal transduction, transcriptional activation, DNA repair, and inhibition of protein degradation (32-35). The

mechanism of this activity and the biologic function of HBxAg in the viral life-cycle remain largely unknown. However, it is well established that HBxAg is necessary for productive HBV infection *in-vivo* and may contribute to the oncogenic potential of HBV. Other functionally important elements within the HBV genome include two direct repeats (DR1 and DR2) in the 5′ ends of the plus strand, which are required for strand-specific DNA synthesis during replication(36). Two enhancer elements, designated as En1 and En2, confer liver-specific expression of viral gene products(37). A glucocorticoid-responsive element (GRE) sequence within the S domain(38), a polyadenylation signal within the core gene, and a posttranscriptional regulatory element overlapping En1 and part of HBxAg ORF have also been described(39).

1.3 HBV INFECTION AND REPLICATION

The HBV replication pathway has been studied in great detail and is summarized in Figure 2. The initial phase of HBV infection involves the attachment of mature virions to host cell membranes, likely involving the pre-S domain of the surface protein(40). HBV initiates infection by binding to a low affinity receptor, a heparan sulfate proteoglycan (HSPG) through the infectivity determinant in the surface-exposed antigenic loop (AGL), a polypeptide present in the S domain common to all envelope proteins (41-44). Glypican-5 (GPC5), a protein associated with proteoglycans, has recently been reported to be involved in entry of HBV and hepatitis delta virus (HDV, a satellite virus of HBV by using HBV envelope proteins for cell entry), providing further clarity on how HSPG mediate HBV entry(45). Proteoglycan binding is followed by a more specific, and higher affinity, interaction of the N-terminal myristoylated peptide in the pre-S1 domain of L protein with the host cellular plasma membrane bearing Sodium Taurocholate Cotransporting Polypeptide (NTCP) receptor (44, 46-49). It has been shown that clathrin-dependent endocytosis is critical for HBV infection through interaction of clathrin associated heavy protein and adaptor (AP-2) with the pre-S1 domain of L protein(50) Following internalization of HBV virions, there is a Rab5-and Rab7-dependent transport through the late endosomal compartment (distinct from recycling endosomes) which is also important to the de-envelopment of capsid (51). Functional caveolin-1 was shown to be necessary for productive HBV infection in HepaRG cells (52). It is speculated that disulfide-reduction within the endosomal compartments is involved in the de-envelopment process as well (53, 54). Nonetheless, de-envelopment must occur, and the de-enveloped virion cargo of partially double stranded viral DNA is delivered to the nucleus. Upon infection, the viral

relaxed circular (rc) DNA genome is transported into the cell nucleus and converted to an episomal cccDNA (44, 55-58) which assembles into a minichromosome and serves as a transcription template for all the viral mRNAs(2). The transcripts from the cccDNA are unspliced, polyadenylated, and possess a 5′ cap structure. The 3.5-kb genomic transcripts consist of two species with different 5′ ends: the pregenomic and the precore RNAs. The pre-genomic (pg) RNA, on getting exported to the cytoplasm, gets packaged into the nucleocapsid. The viral polymerase then reverse transcribes pgRNA into viral minus strand DNA, followed by asymmetric plus strand DNA synthesis to yield the rcDNA genome within the capsid. The mature nucleocapsid is either packaged by viral envelope proteins and secreted as virion particle or redirects the rcDNA to the nucleus to replenish the cccDNA reservoir. The viral envelope proteins can also be secreted alone as subviral particles. A viral precore protein is translated from precore mRNA and further processed in the ER and released as HBeAg through the ER-Golgi secretory pathway (1, 2, 59, 60).

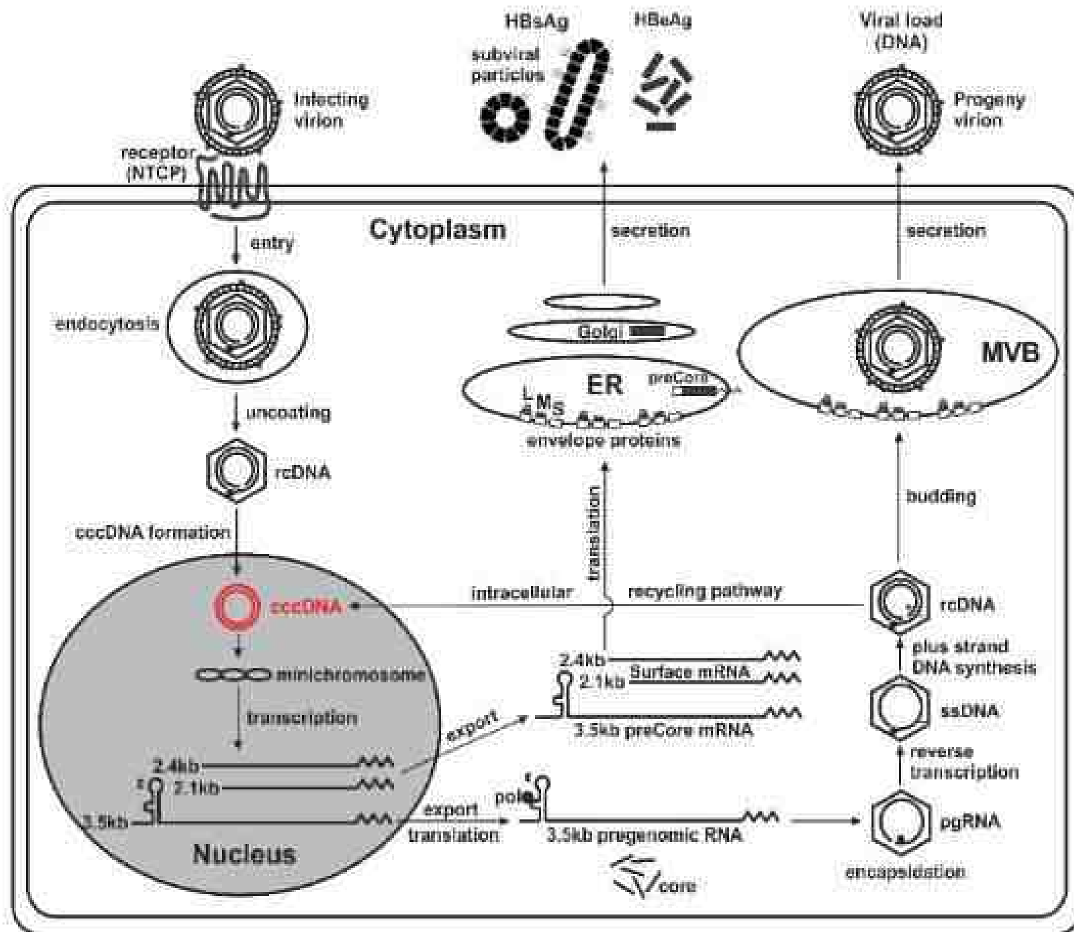


Figure 2: HBV life cycle

Upon infection, the viral rcDNA genome is transported into the cell nucleus and converted to an episomal cccDNA, which assembles into a minichromosome and serves as a transcription template for all the viral mRNAs. The pgRNA, on getting exported to the cytoplasm, gets packaged into the nucleocapsid. The viral polymerase then reverse transcribes pgRNA into viral minus strand DNA, followed by asymmetric plus strand DNA synthesis to yield the rcDNA genome within the capsid. The mature nucleocapsid is either packaged by viral envelope proteins and secreted as virion particle, or redirects the rcDNA

to the nucleus to replenish the cccDNA reservoir. The viral envelope proteins can also be secreted alone as subviral particles. A viral precore protein is translated from precore mRNA and further processed in the ER and released as HBeAg through the ER-Golgi secretory pathway.

1.3.1 HBV cccDNA

cccDNA plays a pivotal role in the HBV life cycle. Once established as a multicopy stable episome in the nucleus of an infected cell, cccDNA functions as the persistent form of the HBV genome that serves as viral mRNA transcription template, and its longevity underlies the chronic nature of HBV infection(61). The longevity of the virus within the host is maintained by this nuclear, episomal, nucleosome-decorated viral DNA called covalently closed circular (ccc) DNA Unfortunately, the major limitation of current medications for chronic hepatitis B is the failure to eliminate cccDNA. The previously reported associations of the core protein with the cccDNA (62-65) highlights the need to unveil the interacting partners of cccDNA that could be used to develop antiviral targets capable of silencing cccDNA. Biosynthesis of rcDNA by reverse transcription of the viral pregenomic RNA is understood in considerable detail, yet conversion of rcDNA to cccDNA is still obscure, fore mostly due to the lack of feasible, cccDNA-dependent assay systems. However, recent literature has provided interesting evidence on involvement of ligases 1 and 3 in cccDNA formation and speculations are employment of host repair mechanism for rcDNA to cccDNA(58).

1.4 HBV GENE EXPRESSION

Four groups of viral RNA are transcribed from cccDNA (Figure 2), namely, - 3.5kb precore mRNA that encodes the precore protein, another 3.5kb pre-genomic(pg) RNA that encodes the core and the polymerase, 2.4kb mRNA for large (L) envelope protein; 2.1kb mRNA for middle (M) and major surface (S) proteins and 0.7kb mRNA for the X protein(2). The pgRNA serves as the template for reverse transcription and the messenger RNA for core and polymerase; the precore RNA directs the translation of the precore gene product. The polymerase translation is initiated at the pol start codon of the pgRNA, probably as a result of a ribosomal scanning mechanism(66). The large HBsAg (L-HBsAg) protein is translated from the 2.4-kb subgenomic RNA, the middle (MHBsAg) and small HBsAg (S-HBsAg) proteins from the various forms of 2.1-kb RNAs, and the HBxAg protein from the 0.7-kb RNA. The S-HBsAg is the major S gene product and the L and M proteins are the minor species. Each surface protein has a glycosylation site in the S domain. L and M proteins occur at the pre-S2 domain with an N-linked oligosaccharide and a myristic acid at the amino-terminal glycine residue of the pre-S1 domain(18). The distribution of the three envelope glycoproteins varies among the types of viral particles, with little or no L and M protein in the 20-nm particles but relatively more L protein in the Dane particles.

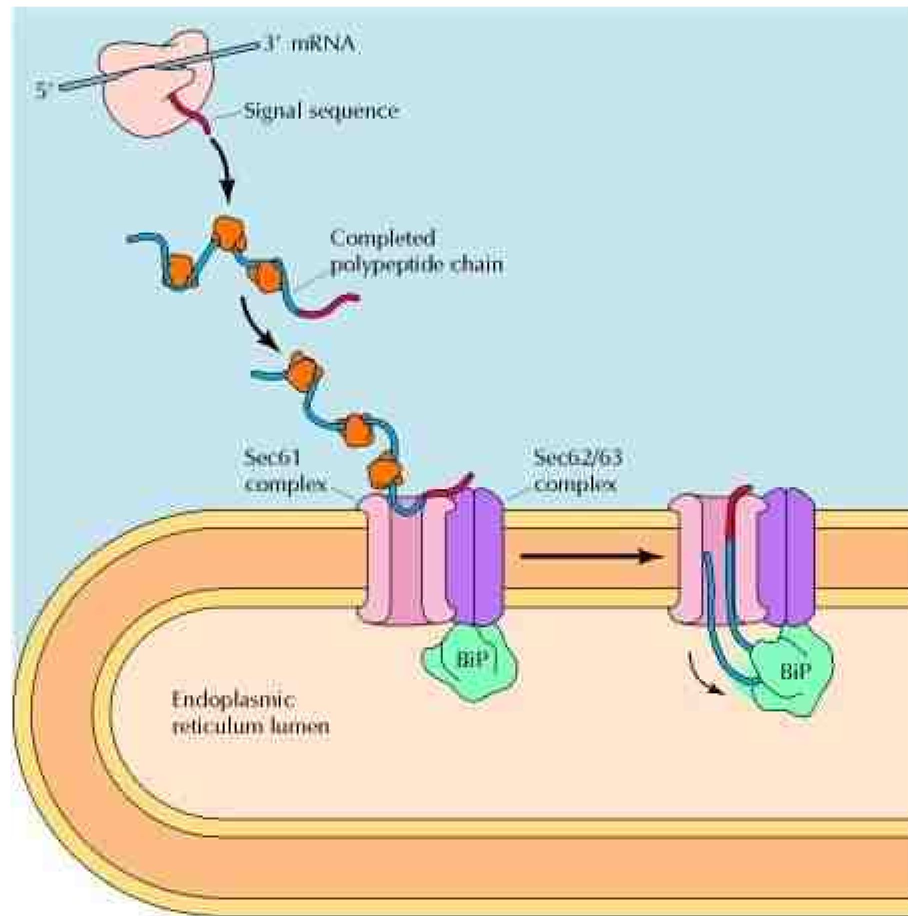
1.4.1 PROTEIN TRANSLATION

The ER has a central role in lipid and protein biosynthesis. Its membrane is the site of production of all the transmembrane proteins and lipids for most of the cell's organelles, including the ER itself, the Golgi apparatus, lysosomes, endosomes, secretory vesicles, and

the plasma membrane. The ER membrane makes a major contribution to mitochondrial and peroxisomal membranes by producing most of their lipids. In addition, almost all of the proteins that will be secreted to the cell exterior—plus those destined for the lumen of the ER, Golgi apparatus, or lysosomes—are initially delivered to the ER lumen. The ER captures selected proteins from the cytosol as they are being synthesized. These proteins are of two types: transmembrane proteins, which are only partly translocated across the ER membrane and become embedded in it (Picture 2), and soluble proteins, which are fully translocated across the ER membrane (Picture 3) and are released into the ER lumen (Picture 4). Some of the transmembrane proteins function in the ER, but many are destined to reside in the plasma membrane or the membrane of another organelle. The soluble proteins are destined either for the lumen of an organelle or for secretion. All of these proteins, regardless of their subsequent fate, are directed to the ER membrane by the same kind of signal sequence and are translocated across it by similar mechanisms. In mammalian cells, the import of proteins into the ER begins before the polypeptide chain is completely synthesized—that is, import is a co-translational process. This distinguishes the process from the import of proteins into mitochondria, chloroplasts, nuclei, and peroxisomes, which are posttranslational processes. Since one end of the protein is usually translocated into the ER as the rest of the polypeptide chain is being made, the protein is never released into the cytosol and therefore is never in danger of folding up before reaching the translocator in the ER membrane. Thus, in contrast to the posttranslational import of proteins into mitochondria and chloroplasts, chaperone proteins are not required to keep the protein unfolded. The ribosome that is synthesizing the protein is directly attached to the ER membrane. These membrane-bound ribosomes coat the surface of the

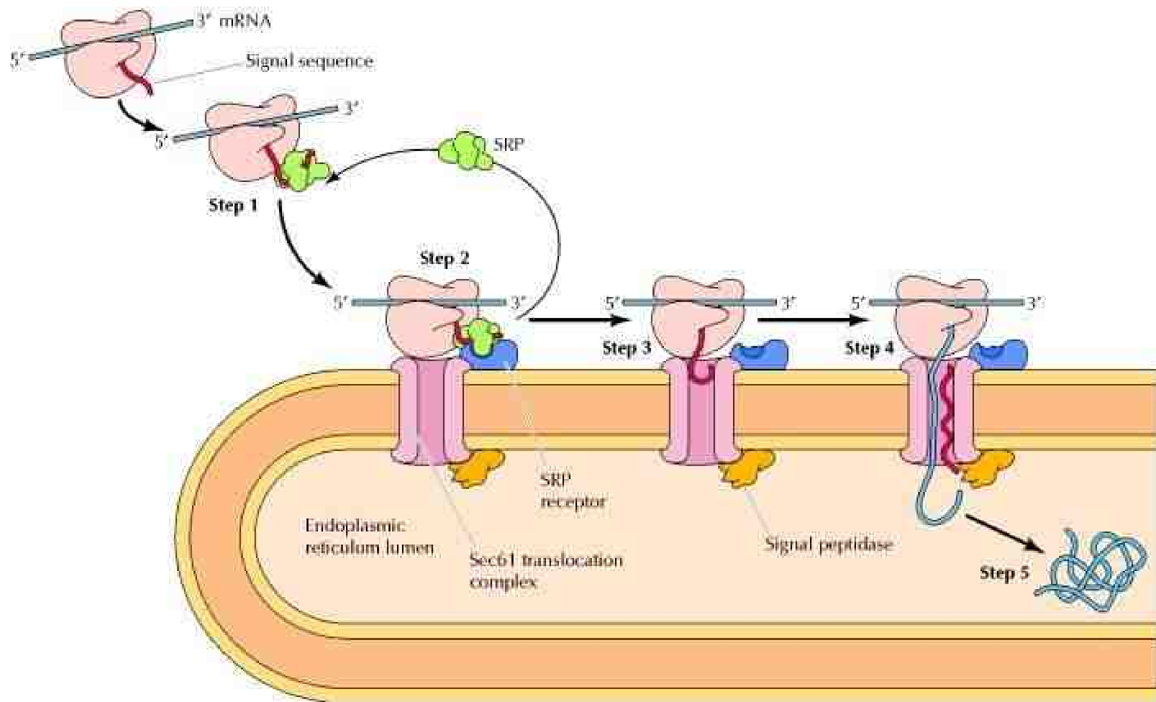
ER, creating regions termed rough ER. There are therefore two spatially separate populations of ribosomes in the cytosol. Membrane-bound ribosomes, attached to the cytosolic side of the ER membrane, are engaged in the synthesis of proteins that are being concurrently translocated into the ER. Free ribosomes, unattached to any membrane, synthesize all other proteins encoded by the nuclear genome. When a ribosome happens to be making a protein with an ER signal sequence, the signal directs the ribosome to the ER membrane. The ER signal sequence is guided to the ER membrane by at least two components: a signal-recognition particle (SRP), which cycles between the ER membrane and the cytosol and binds to the signal sequence, and an SRP receptor in the ER membrane. The SRP binds to the ER signal sequence as soon as the peptide has emerged from the ribosome. This causes a pause in protein synthesis, the pause presumably gives the ribosome enough time to bind to the ER membrane before the synthesis of the polypeptide chain is completed, thereby ensuring that the protein is not released into the cytosol. Once formed, the SRP-ribosome complex binds to the SRP receptor, which is an integral membrane protein exposed only on the cytosolic surface of the rough ER membrane. This interaction brings the SRP-ribosome complex to a protein translocator, Sec 61. The SRP and SRP receptor are then released, and the growing polypeptide chain is transferred across the membrane. The signal sequence in the growing polypeptide chain is thought to trigger the opening of the pore: after the signal sequence is released from the SRP and the growing chain has reached a sufficient length, the signal sequence binds to a specific site inside the pore itself, thereby opening the pore. An ER signal sequence is therefore recognized twice: first, by an SRP in the cytosol, and then by a binding site in the ER protein translocator. This may help to ensure that only appropriate proteins enter the lumen of the ER. The signal

sequence is then cleaved off by signal peptidase as the newly synthesized proteins makes its way into the ER lumen through Sec61 (Picture 3). Translocation of proteins into mitochondria, chloroplasts, and peroxisomes occurs post-translationally, after the protein has been made and released into the cytosol, whereas translocation across the ER membrane usually occurs during translation (co-translationally) (Picture 2) (67).



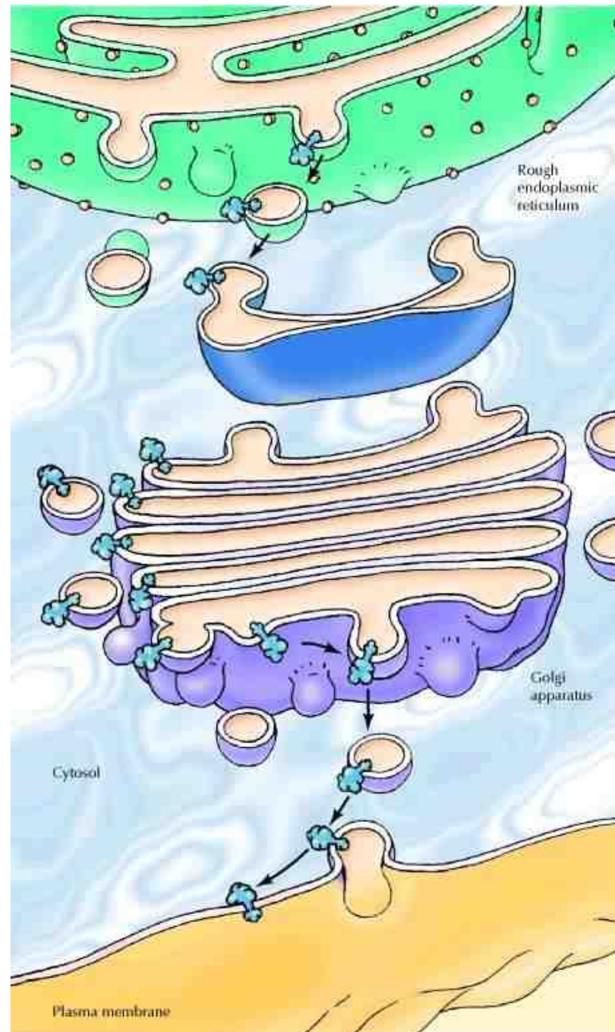
Picture 2: Posttranslational translocation of proteins into the ER

Proteins destined for posttranslational import to the ER are synthesized on free ribosomes and maintained in an unfolded conformation by cytosolic chaperones. Their signal sequences are recognized by the Sec62/63 complex, which is associated with the Sec61 translocation channel in the ER membrane. The Sec63 protein is also associated with a chaperone protein (BiP), which acts as a molecular ratchet to drive protein translocation into the ER (67).



Picture 3: Cotranslational targeting of secretory proteins to the ER

Step 1: As the signal sequence emerges from the ribosome, it is recognized and bound by the signal recognition particle (SRP). Step 2: The SRP escorts the complex to the ER membrane, where it binds to the SRP receptor. Step 3: The SRP is released, the ribosome binds to a membrane translocation complex of Sec61 proteins, and the signal sequence is inserted into a membrane channel. Step 4: Translation resumes, and the growing polypeptide chain is translocated across the membrane. Step 5: Cleavage of the signal sequence by signal peptidase releases the polypeptide into the lumen of the ER (67).



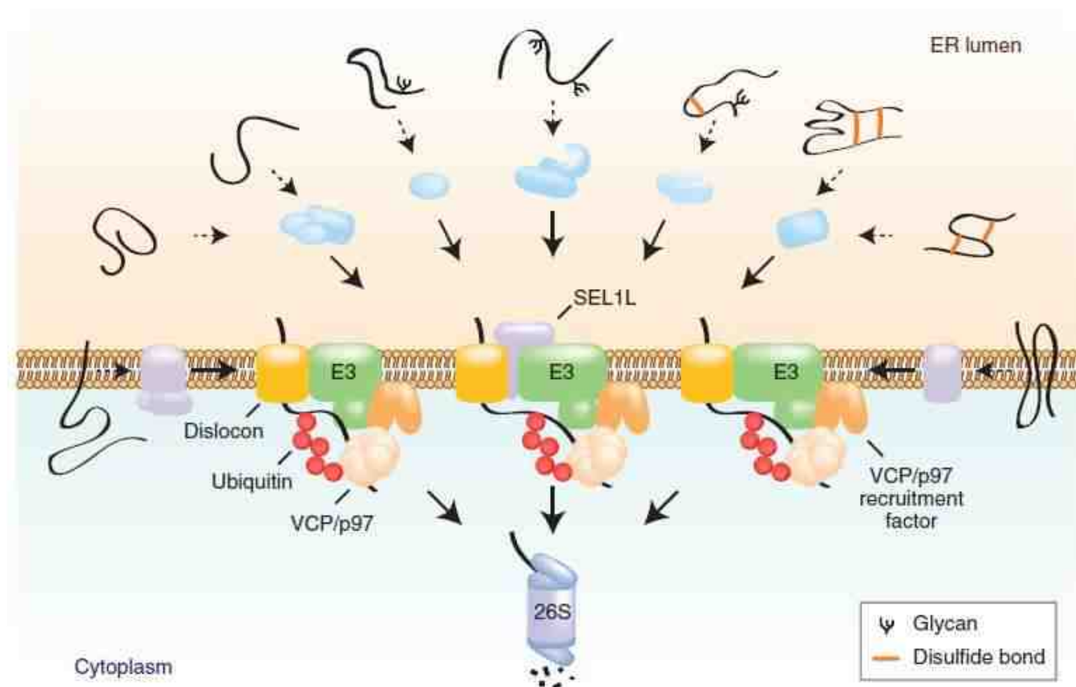
Picture 4: Topology of the secretory pathway

The lumens of the ER and Golgi apparatus are topologically equivalent to the exterior of the cell. Consequently, those portions of polypeptide chains that are translocated into the ER are exposed on the cell surface following transport to the plasma membrane (67).

1.4.2 ENDOPLASMIC RETICULUM ASSOCIATED PROTEIN DEGRADATION

Endoplasmic-reticulum-associated protein degradation (ERAD) designates a cellular pathway which targets misfolded proteins of the endoplasmic reticulum for ubiquitination and subsequent degradation by a protein-degrading complex, called the proteasome (Picture 5). The recognition of misfolded or mutated proteins depends on the detection of substructures within proteins such as exposed hydrophobic regions, unpaired cysteine residues and immature glycans. Because the ubiquitin–proteasome system (UPS) is located in the cytosol, terminally misfolded proteins have to be transported from the endoplasmic reticulum back into cytoplasm. Most evidence suggest that the Hrd1 E3 ubiquitin-protein ligase can function as a retrotranslocon or dislocon to transport substrates into the cytosol(68). Hrd1 is not required for all ERAD events. Since polyubiqitination is essential for the export of substrates, human ubiquitin-binding factors valosin-containing protein (VCP/p97) transports substrates from the endoplasmic reticulum to the cytoplasm with its ATPase activity (69-72). The ubiquitination of terminally misfolded proteins is caused by a cascade of enzymatic reactions. The first of these reactions takes place when the ubiquitin-activating enzyme E1 hydrolyses ATP and forms a high-energy thioester linkage between a cysteine residue in its active site and the C-terminus of ubiquitin. The resulting activated ubiquitin is then passed to an ubiquitin-conjugating enzyme E2. Another group of enzymes, more specifically ubiquitin protein ligases called E3, bind to the misfolded protein. Next they align the protein and E2, thus facilitating the attachment of ubiquitin to lysine residues of the misfolded protein. Following successive addition of ubiquitin molecules to lysine residues of the previously attached ubiquitin, a polyubiquitin chain is formed. A polyubiquitinated protein is produced and this is

recognized by specific subunit in the 19S capping complexes of the 26S proteasome. Hereafter, the polypeptide chain is fed into the central chamber of the 20S core region that contains the proteolytically active sites. Ubiquitin is cleaved before terminal digestion by deubiquitinating enzymes. This third step is very closely associated with the second one, since ubiquitination takes place during the translocation event. However, the proteasomal degradation takes place in the cytoplasm (73-76).



Picture 5: Endoplasmic Reticulum Associated Degradation

Protein adaptors within the ER membrane link substrate recognition to the dislocation apparatus. ERAD substrates (black) differ in topology, the features and location of the folding lesion, and posttranslational modification. To accommodate this diversity, the ERAD system is organized with lumenal substrate recognition factors (blue) and membrane-embedded adaptor proteins (purple) that cooperate to recruit ERAD substrates to a set of E3 ligase-coupled dislocation complexes. The dislocation complexes integrate the coupled processes of substrate ubiquitination, membrane extraction via VCP/p97, and proteolytic destruction by the 26S proteasome (67).

1.4.3 HBeAg BIOGENESIS

HBeAg is a 17kDa protein that is detectable in serum and is correlated to high levels of viremia (77). HBeAg is derived from the precursor protein, p25. The preCore/core ORF contains two in-frame initiation codons that translates into p25 and core protein, respectively (Figure 3). Its coding sequence contains an extension of 29 codons (the "precore" region) at the amino terminus of the protein this region was found to be evolutionarily conserved among mammalian and avian HBVs, suggesting it has functional importance, although at least for duck HBV it has been shown to be nonessential for replication of infectious virion. Using in vitro assays for protein translocation across the ER membrane, Garcia et al showed that that the precore region of the HBV genome encodes a signal sequence that is recognized by signal recognition particle, which targeted the nascent precore protein to the endoplasmic reticulum membrane (190). A 19-amino acid signal peptide was removed by signal peptidase on the lumenal side of the microsomal membrane, generating the p22 protein with 10 additional amino acids at its amino terminus(78). p25 translation on precore mRNA starts at the first ATG site while translation of the core protein on pgRNA starts at the second ATG. In contrast to the core protein, which spontaneously oligomerizes into the viral capsid, the p25 protein is a 25kDa monomer with a 29 amino acid long N-terminal extension. The initial 19 amino acid signal peptide guides the precore mRNA to translocate to ER membrane, then the signal peptide sequence is co-translationally cleaved off within the ER lumen resulting in the precore (p22) protein. This 22kDa precore protein is either translocated into the ER or released back to the cytoplasm(77). *Duriez et al.*, using human cells expressing precore and a convenient fractionation assay, demonstrated that the cytosolic form is identical to the ER

form and retro-transported in the cytoplasm through the ER-associated degradation pathway. They showed that the precore escapes proteasome due to its low lysine content and accumulates in the cytosol. In the presence of precore, they found a specific redistribution of the Grp78/BiP chaperone protein to cytosol and demonstrated a specific interaction between precore and Grp78/BiP(79). Due to the presence of nuclear localization signal (NLS) on precore, this protein can also be transported into nucleus (80). Newly-formed p22 translocates to the Golgi and in the *trans*-Golgi network, the arginine-rich C-terminus is cleaved by furin endoprotease to release the 17kDa mature HBeAg into the blood in a dimeric form (81-83) (Figure 3, 4).

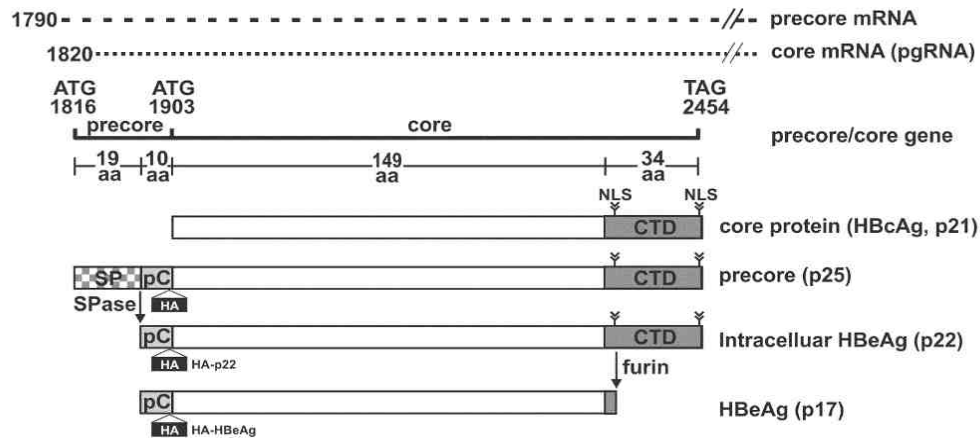


Figure 3: Core, precore and HBeAg transcription

Core protein is translated from pre-genomic mRNA, using the ATG codon at 1903 as initiation site. HBeAg is translated from the precore mRNA, using an upstream in-frame ATG at 1816. Precore mRNA on translation gives p25 as a protein product that is cleaved at its N-terminal (19 amino acids signal peptide) by a peptidase during translocation to ER giving the p22 or precore. Further cleavage of the CTD by furin in the *trans*-Golgi network generates HBeAg that is released into the serum.

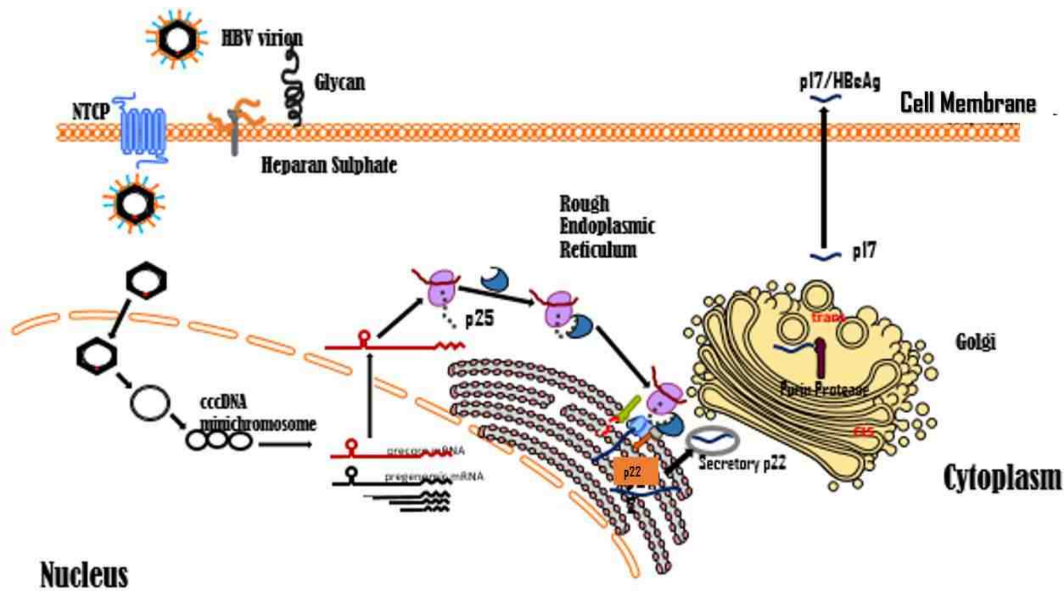


Figure 4: Biogenesis of p22 and HBeAg

cccDNA transcription leads to production of precore mRNA among other mRNAs which is transported to the cytoplasm. The precore mRNA starts to be translated and as the signal peptide is translated, the SRP complex binds to it and stalls further translation. The SRP-mRNA-ribosome complex is transported to SRP receptor on the ER lumen leading to the opening Sec31 translocon and cleavage of the signal peptide by the signal peptidase followed by the translation of the rest of the precore mRNA forming the p22 protein. A part of the p22 protein follows the ER-Golgi secretory pathway where the furin protease cleaves off the CTD of p22 in the Golgi to form HBeAg/p17 that is secreted out into the serum. Rest of the newly formed p22 retro-translocates into the cytosol and also translocates to the nucleus.

1.5 INNATE IMMUNITY IN HBV

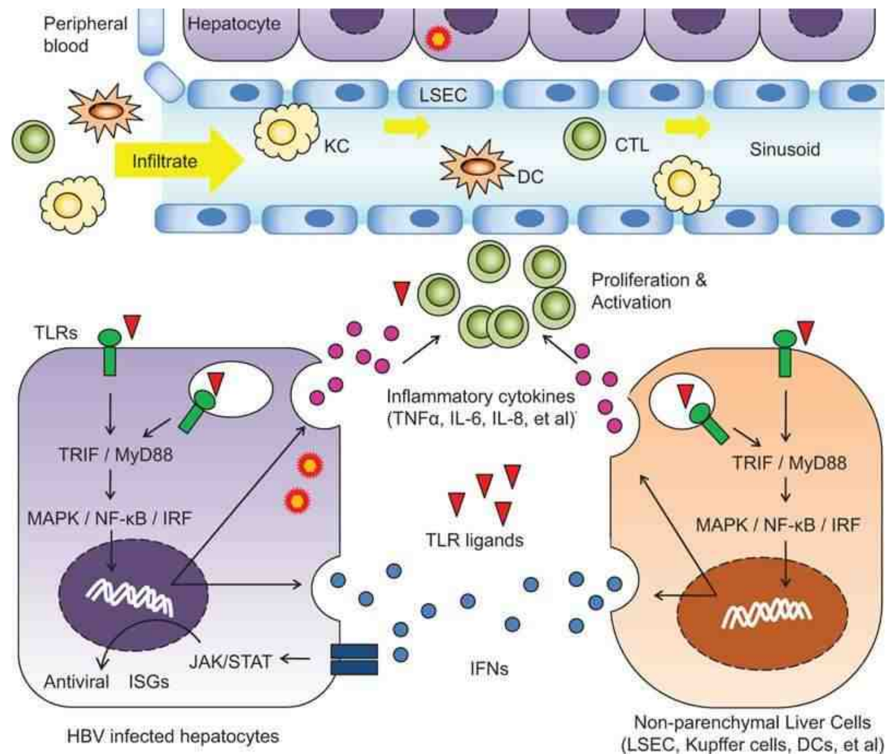
The liver has been increasingly recognized as an immunological organ with innate immune features. Intrahepatic lymphocytes exert a distinct composition and phenotype characteristics, and are enriched in NK cells, NKT cells, and macrophages (i.e., Kupffer cells), which constitute the innate immune system. When activated, NK cells and NKT cells secrete high levels of proinflammatory and anti-inflammatory cytokines, which play major roles in resistance of viral infection and regulation of innate and adaptive immune responses. In liver, PRRs, particularly TLR and RIG-I, are widely expressed in/on both parenchymal and nonparenchymal cells. Once PRR identifies a pathogen associated molecular pattern (PAMP), the signal pathways are activated and the effector cell (such as NK and NKT cell) is triggered to perform its functions immediately. The activated signal pathways include the activation of interferon regulatory factor 3 (IRF3) or IRF7 and nuclear factor kappa B (NF-κB),leading to the induction of type I IFN and the expression of a variety of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-6, IL-1β, and IL-18. However, activation of NK and NKT cells has also been linked to liver injury in HBV transgenic mice, due to their ability to secrete proinflammatory cytokines in the hepatic microenvironment(84). The involvement of NK activation in liver injury was not observed in human studies. On the contrary, one study comparing NK cell function in patients with chronic HBV infection with those from chronic HCV infection and healthy controls showed a more pronounced functional defect in chronic HBV infection than in the other two groups(85). One recent study showed that NK numbers and cytotoxic function are maintained in peripheral blood of patients with chronic HBV infection compared to healthy controls, but their activation and cytokine producing abilities (IFN-γ and TNF-α)

are impaired(86). Overall, these studies indicate that the main function of NK and NKT cells is to limit viral replication via cytokine production rather than direct cytotoxicity and that function is compromised during chronic HBV infection, which may contribute to viral persistence.

HBV did not cause any change in genes associated with innate immune responses during acute HBV infection of chimpanzees(87). The authors concluded that HBV did not induce an intrahepatic innate immune response that could be detected in the infected animals because it acts like a stealth virus early in infection, remaining undetected and spreading until the onset of the adaptive immune response several weeks later. However, the mechanisms by which HBV accomplishes this task are less understood. Interestingly, HBV replication is sensitive to the suppressive effects of interferons in studies using the transgenic mouse model or hepatoma cell lines. HBV was shown to replicate in IFNγ KO and IFNα/β receptor KO mice at levels higher than those observed in control mice, implying that baseline levels of these cytokines control HBV replication in the absence of inflammation(88). Also, expression of adaptor proteins TRIF and MyD88 (TLR adaptor proteins) and of IPS-1 (RIG-I/MDA5 adaptor protein) in human hepatoma cells was enough to limit HBV replication by reducing the steady-state levels of viral mRNAs, in a manner dependent on NF-κB transcription factor(89). The importance of TLR receptor signaling in controlling HBV replication was confirmed in another study showing that a single intravenous injection of ligands specific for TLR3 (TRIF only), TLR4 (TRIF and MyD88), TLR5 (MyD88 only), TLR7 (MyD88 only), and TLR9 (MyD88 only) was efficient to inhibit HBV replication in a noncytolytical and IFNα/β dependent manner in

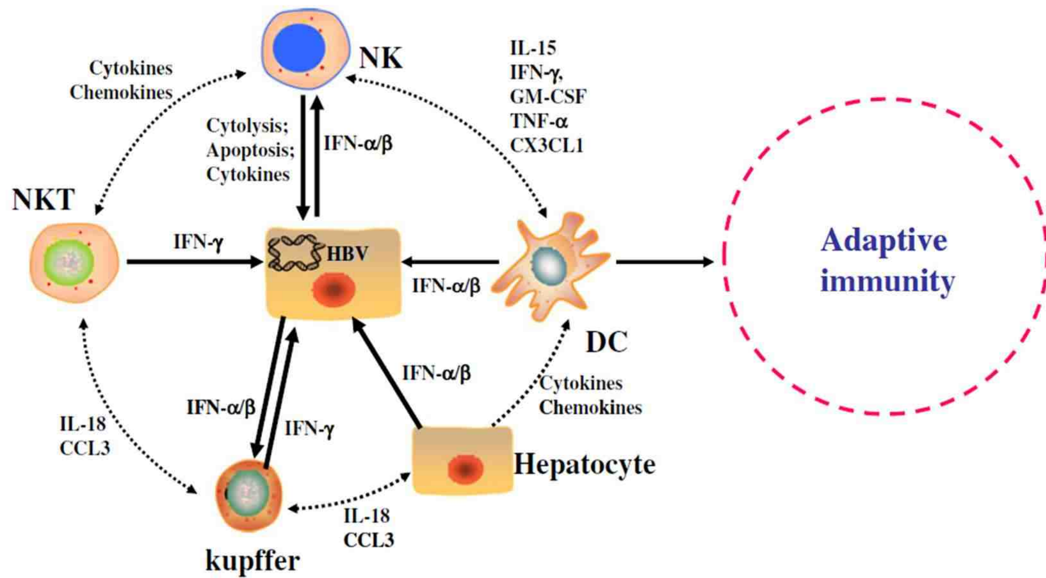
HBV transgenic mice(90). Picture 6 outlines the TLR and RLR-mediated immune response to HBV in infected cells.

Another characteristic of the interferon response in HBV infection is that CD8+ T cells seem to control the viral replication through an IFN-γ dependent mechanism rather than direct cell killing of infected hepatocytes(91). The importance of noncytolytic T cell functions was shown in a study where the onset of viral clearance in HBV infected chimpanzees was tightly associated with the appearance of virus-specific T cells and mRNA in the liver, before the onset of hepatocyte destruction and clinical hepatitis(92). These results suggest that direct cytological cell killing and IFN-γ secretion are functionally distinct mechanisms of CD8+ T cells that account for control of hepatocyte destruction and viral replication, respectively(93). Picture 7 outlines the known mechanisms by which innate immunity controls HBV in infected cells.



Picture 6: TLRs- or RLRs-mediated immune responses to HBV

Upon stimulation with their ligands, signaling pathways are activated. TLRs use their adaptors- the TIR-domain-containing adaptor-inducing IFN-β (TRIF) or the myeloid differentiation factor-88 (MyD88), which activates a cascade that involves interleukin-1 receptor-associated kinase (IRAK-1), tumor-necrosis-factor receptor-associated factor 6 (TRAF6) and nuclear factor-kB (NF-kB). Signaling from TLRs also activates the IFN regulatory factors (IRFs), in particular IRF3 and IRF7, leading to type I IFN production. As for RIG-I, viral RNA or 5′-triphosphate-siRNA binding to the C-terminal helicase domain of RIG-I lead to a conformational change and exposure of the CARD domains, and subsequently activate IFN promoter stimulator 1 (IPS-1), IRF3, and NF-κB, leading to the production of type I IFN and inflammatory cytokines. HBx, HBsAg or polymerase exert mechanisms to disturb the activation of TLR or RIG-I pathway (187).



Picture 7: Control of HBV infection by innate immunity

NK cells, NKT cells, DC cells, Kupffer cells and even hepatocytes have all been shown to play important roles in defense against HBV. Briefly, IFN-α/β recruits and mediates the activation of Kupffer cells which in turn produce IL-18 and the chemokine CCL3, which improve the function of NK and NKT cell activity. More importantly, the NK cell is a critical player in helping DC cells to stimulate adaptive immunity against HBV infection. Hepatic environment mounted by chronic HBV infection is associated with functional impairment of NK cells with reduced number and low production of antiviral cytokines which correlates to the reduced ability of DCs to prime T cells.

1.5.1 VIRAL STRATEGIES TO COUNTERACT HOST INNATE IMMUNE DEFENSES

Recent developments in our understanding of the HBV infection have shown that the virus is not just a stealth pathogen, but that it employs active mechanisms to avoid recognition by the innate immune system. This may explain previous observations on its apparent inability to be detected by PRRs. HBV employs different mechanisms to counteract the host interferon response in infected cells as outlined in Table 1.

Table 1: Summary of the mechanisms employed by HBV to counteract induction of an efficient interferon response in infected cells

Experimental Model	Mechanism	HBV Protein Involved	Functional Outcome	Reference
Human hepatocyte cell line	Inhibition of IRF3	HBV polymerase	Inhibition of IFN-β induction	(94)
Hepatoma cell lines, liver tumor samples	MAVS degradation	HBx	Prevention of IFN-β induction	(95)
Hepatoma cell lines	Blocking DDX3 DEAD box RNA helicase	HBV polymerase	Inhibition of IFN-β promoter in response to RIG-I/MDA5 and TLR3	(96)
Hepatoma cell lines, HBx transgenic mouse	HBx binding to IPS-1	HBx	Inhibition of IFN-β induction	(97)
Hepatoma cell lines	Binding and inactivation of mitochondrial membrane protein virus-	HBx	Inhibition of virus-triggered IRF3 activation and IFN-β induction	(95)

	induced signaling adaptor (VISA)			
HBV transgenic mice and hepatoma cell lines		HBsAg, HBeAg, or HBV virions	Suppression of TLR 1-9 induced activation of IRF3, NF-κB, ERK1/2 and IFN-β production	(98)
VSV infection of hepatoma cell lines and monocyte-derived dendritic cells from patients with HBV infection			Reduced RIG-I expression and IFN-β production	(99)

The role of innate immunity in controlling HBV replication has been neglected for a number of years due to results from studies with HBV infected animals that failed to detect induction of type 1 interferon responses following HBV challenge. The ability of antibody responses to HBV surface antigen to protect adults from HBV challenge also indicated the importance of adaptive immunity in controlling viral replication. However, adaptive immunity alone cannot explain the persistence of HBV during chronic infection and the immune pathogenesis that characterizes chronic hepatitis B. A better understanding of how the immune systems works has led to a reconsideration of the role of innate immunity in HBV infection. Recent research studies mentioned in this review have shown a different image of HBV pathogenesis, with active mechanisms aimed at inactivating various components of the innate immune system. Thus, the lack of interferon responses

during acute infection seems to be the result of inactivation of various signaling pathways that normally induce IFN production in other viral infection. HBx and HBV polymerase are the proteins associated most frequently with inactivation of the TLR and RIG-I pathways and ultimately with impaired IFN production. These mechanisms may constitute important factors of viral persistence during natural infection, since in vitro studies have shown that HBV is sensitive to antiviral properties of IFNs. During the course of infection HBV also contributes to a sustained immunosuppressive state that may favor its replication, since the number of Tregs increase as disease progresses, which also decreases the number and function of HBV specific T lymphocytes(100).

1.6 FUNCTIONS OF HBeAg

Precore is a precursor of HBeAg and hence referred to as intracellular HBeAg. As an auxiliary protein of HBV, HBeAg is not a component of viral and subviral particles, the precise function of precore/HBeAg in HBV life cycle is currently unknown. It is ,however, known that precore is not required for HBV DNA replication in plasmid-based transient and stable transfections (59, 77) but whether precore or its derivatives play a role in cccDNA metabolism and transcription is unknown. The precore form shares the same sequence as the core except for the additional 10 amino acids long precore region at the N-terminus that bears a cysteine (that is absent in the core). This -7Cys can form intramolecular disulfide bond with +61Cys (present in the core that helps it to form intermolecular disulfide bond during capsid formation) preventing precore proteins from forming capsid structures (101). In view of the fact that HBeAg is not a structural component of the virus and is not required for viral replication, the function of HBeAg/precore is a candidate for modification of host functions, such as anti-HBV immune responses.

In the clinic, HBeAg is generally used as a surrogate marker for high levels of viral replication(2). As stated above, HBeAg is a product of the precore gene and transcript, which is derived directly from cccDNA. Hence, the level of HBeAg in the circulation correlates, in general, with viremia and HBeAg positivity is considered a strong predictor of cirrhosis and Hepatocellular Carcinoma (HCC)(102). Loss of detectable HBeAg in individuals with chronic viral hepatitis and the appearance of detectable levels of antibody to HBeAg is usually considered to be a beneficial milestone and evidence of reduced viral replication (103).

1.6.1 HBeAg AND IMMUNITY

Although there are studies indicating that HBV is able to activate IFN production upon infection of hepatocytes(104, 105), HBV is currently viewed as a stealth virus that can establish itself efficiently by evading the host innate immune responses leading to a chronic infection (106, 107). In line with this notion, it has been reported that HBV particles themselves can readily inhibit host innate immune responses(107). When infection is established, HBV polymerase and X protein have been shown to antagonize cytoplasmic pattern recognition receptors (PRRs)-elicited signaling cascades and block the subsequent production of IFN and other cytokines (95, 97, 108-112). In addition, previous studies have showed that the HBeAg suppresses the TLR2 and IL-1 signaling pathways (85, 113, 114), suggesting that this viral protein is also a key regulator of antiviral innate immune responses.

Despite the fact that HBV blocks IFN production, the cytokine is clinically used to treat hepatitis B patients, but only approximately one third of the patients respond to IFN-α treatment(115). This limited antiviral activity of IFN-α against HBV could be due to the blockade of IFN-elicited JAK-STAT pathway by a HBV protein, which has been supported by a study showing that HBV prevents the nuclear translocation of STAT1 and inhibits interferon stimulated gene (ISG) expression(116). However, which viral protein(s) inhibits IFN signaling needs further investigation. Interestingly, it was found that patients with low pre-treatment HBeAg levels are more likely to respond to IFN-α therapy(117, 118), suggesting that HBeAg or its intracellular precursor may alter the IFN signaling. A recent study by *Tian et al* has identified maternal HBeAg as a factor altering macrophage function in offspring to drive viral persistence upon vertical transmission and also affecting the

adaptive immunity response to HBV(119). In the clinic, HBeAg positivity is generally used as a surrogate marker for high levels of viral replication. Loss of detectable HBeAg in individuals with chronic viral hepatitis and the appearance of detectable levels of antibody to HBeAg is usually considered to be a beneficial milestone and/or evidence of reduced viral replication, suggesting a role of HBeAg in HBV persistence(111). In line with this, previous studies have demonstrated that the circulating HBeAg elicits T cell tolerance and depletes inflammatory HBeAg- and HBcAg-specific Th1 cells that are necessary for viral clearance(95, 109), and the HBV core-specific CTL responses are significantly weaker in HBeAg(+) than HBeAg(-) patients(112). During HBV vertical transmission, more than 90% of untreated infants born to HBeAg(+) mothers become chronically infected due to T cell immune tolerance to HBV infection(97, 108, 110). A mechanistic study in HBV transgenic mice revealed that such impairment of CD8+ T cell responses is attributed to the maternal HBeAg-mediated upregulation of PD-L1 in fetal liver macrophages(85). HBeAg, thus, may represent a viral strategy to persist in the host through inducing immune tolerance and/or exhaustion. In terms of innate immunity, it has been reported that HBeAg downregulates NK cell-mediated IFN-γ production and IL-18 signaling(113), and precore protein suppresses the TLR2 and IL-1 signaling pathways intracellularly(114, 116, 117). However, another study demonstrated that HBeAg triggers IL-1 response through binding to IL-1 receptor accessory protein(119).

1.6.2 HBeAg SEROCONVERSIONS AND MUTATIONS

The most common precore mutant virus has a point mutation from G to A at nucleotide 1896 (G1896A), which creates a stop codon 28 and abolishes synthesis of

HBeAg(120). The double mutations in the basal core promoter region at nucleotide 1762 (A–T) and 1764 (G–A) are associated with reduced synthesis of HBeAg by suppressing the transcription of precore mRNA by 50%–70% (121, 122). Although the association between precore and basal core promoter mutations with HBeAg-negative chronic hepatitis B is well established (123-125), their presence in HBeAg-positive chronic hepatitis B is much less appreciated. Precore and/or basal core promoter mutations were found in approximately one-half of HBeAg-positive patients with chronic hepatitis B(124, 125). In recent years, the clinical significance of precore and basal core promoter mutations in HBeAg-positive patients has been studied with regard to spontaneous HBeAg seroconversion, but little is known regarding the potential role of these variants in treatment-associated HBeAg seroconversion (126-130).

During spontaneous HBeAg seroconversion, the prevalence of both the precore mutation G1896A and basal core promoter mutations A1762T/G1764A appears to increase in patients who experience HBeAg seroconversion. The prevalence of these mutations is also increased in patients with persistently or intermittently elevated ALT levels, compared with immune tolerant patients with persistently normal ALT levels(131). None of these studies, however, examined the association between the presence of precore and basal core promoter mutations and HBeAg seroconversion with complete HBV DNA suppression (i.e., undetectable HBV DNA by PCR techniques)(132).

Finally, the association between precore and basal core promoter mutations with HBV genotypes should also be noted. Most of the literature concerning this association comes from Asia, where HBV genotypes B and C are the primary genotypes. In general, precore mutation A1896 is known to be more common in patients with HBV genotype B

than in patients with HBV genotype C, whereas the basal core promoter mutation T1762/A1764 is more common in patients with HBV genotype C (131, 133-135). Precore and, especially, basal core promoter mutations have been shown to be risk factors for HCC, independent of HBV genotype status, age, sex, and HBV DNA level (OR, 2.4 [95% CI, 1.1–5.3] and 12.8 [95% CI, 5.9–27.8], respectively)(136, 137). The presence of precore and basal core promoter mutations is associated with a higher risk of advanced liver disease in HBeAg-positive chronic hepatitis B than in HBeAg-negative chronic hepatitis B(130, 132).

1.7 HEPATITIS B TREATMENTS

While an efficient prophylactic vaccine is available(138), there are currently seven medications approved by the United States (US) Food and Drug Administration (FDA) for management of chronic HBV infection, including two IFNs (standard and PEGylated IFN-α) and five nucleos(t)ide analogue polymerase (pol) inhibitors. Pegylated IFN and nucleos(t)ide analogues (lamivudine, adefovir, entecavir, tenofovir disoproxil, and tenofovir alafenamide) suppress HBV DNA replication and improve liver inflammation and fibrosis. Long-term viral suppression is associated with regression of liver fibrosis and reduced risk of HCC in cohort studies. The cure (defined as hepatitis B surface antigen loss with undetectable HBV DNA) rates after treatment remain low (3%-7% with pegylated IFN and 1%-12% with nucleos[t]ide analogue therapy). Pegylated IFN therapy can be completed in 48 weeks and is not associated with the development of resistance; however, its use is limited by poor tolerability and adverse effects such as bone marrow suppression and exacerbation of existing neuropsychiatric symptoms such as depression. Newer agents (entecavir, tenofovir disoproxil, and tenofovir alafenamide) may be associated with a significantly reduced risk of drug resistance compared with older agents (lamivudine and adefovir) and should be considered as the first-line treatment. Antiviral treatment with either pegylated IFN or a nucleos(t)ide analogue (lamivudine, adefovir, entecavir, tenofovir disoproxil, or tenofovir alafenamide) should be offered to patients with chronic HBV infection and liver inflammation in an effort to reduce progression of liver disease. Nucleos(t)ide analogues should be considered as first-line therapy. Because cure rates are low, most patients will require therapy indefinitely(7). Due to severe side effects, only a fraction of patients are eligible for IFN therapy, and <10% of them show a sustained

virological response, measured as loss of HBsAg (139). NAs are much better tolerated, and the most potent drugs, entecavir and tenofovir, can reduce viremia by 5–6 logs, often below detection limit, and with low rates of viral resistance development(140). However, HBsAg clearance is very rare (0–5%) even after prolonged treatment (139) and the frequent viral rebound upon therapy withdrawal indicates a need for lifelong treatment(141). Reactivation can even occur, upon immunosuppression, in patients who resolved an acute HBV infection decades ago(142) indicating that the virus can be immunologically controlled but is not eliminated. Current therapies rarely achieve a cure due to the refractory nature of an intracellular viral replication intermediate termed ccc DNA(65). Therefore, development of novel direct acting agents (DAAs) and host targeting agents (HTAs) against HBV infection is warranted to enrich the landscape of HBV antivirals. Several chemotypes of small molecules, including heteroaryldihydropyrimidines (HAPs) (such as, Bay 41–4109 and GLS4)(143, 144); sulfamoylbenzamides (SBAs) and phenylpropenamides (PPAs), represented by ENAN-34017 and AT-61,(145, 146) respectively, have been discovered to allosterically modulate core protein structure and consequentially alter the kinetics and pathway of core protein assembly, resulting in formation of irregularly-shaped core protein aggregates or "empty" capsids devoid of pgRNA and viral DNA polymerase. Due to their unique structures and essential roles in viral replication, disruption of, or interference with, nucleocapsid assembly and/or disassembly with small molecular core protein allosteric modulators (CpAMs) represents a new frontier in development of novel antiviral agents against HBV(147, 148). Over the last two decades, at least five chemotypes of CpAMs have been reported (148). A recent report showed that HAPs and SBAs, but not PPAs, induce disassembly of nucleocapsids

from virions as well as double-stranded DNA-containing cytoplasmic progeny nucleocapsids and consequentially interfere with cccDNA biosynthesis from *de novo* infection and intracellular amplification pathways(149).

1.8 QUESTIONS AND HYPOTHESES

Based on the current knowledge of Hepatitis B virus infection and mechanisms used by the virus to persist within the host and the presence of the p22 protein with its function(s) being elusive, we present the following questions and hypotheses.

1.8.1 QUESTIONS

Question I: How similar and different are p22 and HBV core proteins in their capsid-forming ability?

Question II: How does p22 retro-translocate to cytosol from ER lumen

Question III: Is p22 playing a role in the suppression of the innate immune defenses of the host against HBV infection?

Question IV: Does nuclear p22 interact with cccDNA minichromosome?

1.8.2 HYPOTHESES

Hypothesis I: The 10aa extended NTD of p22 prevents it from forming a capsid unlike core

Hypothesis II: p22 is using ERAD pathway for its retro-translocation to cytosol

Hypothesis III: p22 negatively affects the IFN-elicited JAK-STAT signaling by blocking the signaling

Hypothesis IV: p22 interacts with cccDNA minichromosome through its CTD and regulates the stability and/or transcription of cccDNA within the nucleus

CHAPTER 2

MATERIALS AND METHODS

2.1 PLASMIDS

HBV replication-competent plasmid pHBV1.3 that contains a 1.3-mer replicon of HBV genome, and pCMVHBV that transcribes HBV pgRNA under the control of a human cytomegalovirus immediate-early (CMV-IE) promoter, (150-154). The core-null HBV plasmid pHBV1.3ΔC is a 1.3-mer HBV plasmid with mutation of the start codon (ATG to ATA) of core protein ORF (155) (provided by Dr. Robert Lanford). The precore/HBeAg-null HBV plasmid pCMVHBVΔe was made by replacing the sequence between RsrII and AflII restriction sites on pCMVHBV with a synthetic sequence containing the precore start codon mutation (AUG to TTG). The plasmid pTREHBV-HAe supports the replication of HBV genome under an inducible CMV promoter, it has been previously used together with plasmid pTet-off (Clontech) to generate the HepBHAe82 stable cell line which produces HA-tagged HBeAg (HA-HBeAg) in a cccDNA–dependent manner(156). Plasmid pTREHBVΔHAe was made by replacing the sequence between SacI and BspEI restriction sites on pTREHBV-HAe with a synthetic sequence containing the precore stop codon mutation (G1896A). HBV protein expression plasmids, including the pcHBc expressing HBV core protein, the pcHBe (a.k.a. p22) encoding HBV precore and HBeAg, the pcHA-HBe (a.k.a. HA-p22) expressing the HA-tagged HBV precore and HBeAg, have been described in our previous publication(156). Plasmid pcHBeΔSP was constructed by deleting the N-terminal signal peptide (SP, aa 2-19) coding sequence from pcHBe. Plasmid pcHBe-C-7Q expressing precore 396 with the cysteine (C) of aa -7 in precore domain being changed to glutamine (Q) was made through site-directed mutagenesis of pcHBe. Plasmid

HA-p22ΔCTD was constructed by deleting the HBV precore/core C-terminal domain (CTD, aa 149-183) coding sequence from plasmid HA-p22. Plasmid pLMS expressing HBV large (L), middle (M), and small (S) envelope proteins was provided by Dr. Youhua Xie(157). Plasmid pCMV-FLAG-Pol expressing 3×FLAG-tagged 401 HBV Pol was provided by Dr. Wang-Shick Ryu(158). Plasmid FL1-154 HBx expressing FLAG-tagged HBx was provided by Dr. Michael Bouchard(159). Firefly luciferase reporter plasmid ISG56-Luc was provided by Dr. Kui Li (160). Plasmid expressing N-terminal FLAG-tagged karyopherin-α1-6 (FLAG-Kα1-6) and plasmid expressing HA-tagged Ebola VP24 (HA-VP24) were provided by Dr. Christopher Basler through BEI Resources(161, 162). The plasmid description and respective references may also be found in Table A2.

2.2 CELL LINES

HepG2 and 293T cells were purchased from ATCC and cultured in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The tetracycline-inducible HBV stable cell line HepBHAe82 was established previously(156), and maintained in the same way as HepG2, except for the addition of 1 μg/ml 411 tetracycline (tet) and 400 μg/ml G418. When required, the culture medium was switched to tet-412 free to initiate HBV replication and cccDNA-dependent HA-HBeAg expression in HepBHAe82 413 cells. The HepHA-HBe4 stable cell line that constitutively expresses HA-HBeAg(156), and the HepG2-NTCP12 cells that support HBV infection(163), were described previously. PXB cells were obtained from Phoenix Bio and maintain in modified dHCGM (164, 165) prepared by adding supplements to DMEM.

2.3 TRANSFECTION

Cells were seeded in the collagen coated plate and were transfected with indicated plasmid(s) by Lipofectamine 3000 (Life Technologies) according to the manufacturer's directions.

2.4 HBV RNA AND DNA ANALYSES

HBV total RNA, cytoplasmic encapsidated pgRNA, and core DNA were isolated and detected by Northern and Southern blotting as previously described (58, 154, 166). Quantitative PCR (qPCR) analysis of HBV core DNA was conducted according to literature(163).

2.5 PREPARATION OF VIRUS INOCULUM

HepG2 cells were transfected with wild-type pCMVHBV or precore-null pCMVHBVΔe; or with pTREHBV-HAe or mutant precore-null pTREHBVΔHAe plasmid, plus pTet-off and pLMS in 2:1:2 ratio, and the cells were maintained in tetracycline-free media post transfection. Supernatant from the transfected cells were collected every other day for over an 8-day period. The pooled supernatant was mixed with polyethylene glycol (PEG)-8000 powder (final concentration of 10%) and gently rotated at 4°C for overnight, HBV particles were then precipitated by centrifugation at 1,000×g for 20 min at 4°C and re-dissolved in serum-free DMEM/F12 medium with 1% volume of the original supernatant samples. The concentrated virus stocks of wild-type or two different kinds of precore-null viruses (G1896A and A1814T) were aliquoted and stored at -80°C.

HBV virus genome equivalent (vge) was determined using HBV core DNA qPCR and a standard curve using titrated HBV plasmid (163).

2.6 HBV INFECTION

HepG2-NTCP12 cells were seeded into collagen-coated 96-well plates with density of 6×10^5 cells/well and cultured in regular DMEM/F12 medium for overnight, and then the culture medium was switched to Cellartis hepatocyte maintenance medium (Takara). After 24 h, the cells were incubated with HBV inoculum at 500 viral genome equivalent (VGE) per cell in PMM containing 4% PEG-8000. The cells with HBV inoculation were transferred to 37°C CO_2 incubator. 24 h later, the HBV inocula were removed and the infected cells were maintained in regular primary hepatocyte maintenance medium (PMM), specifically Williams E medium supplemented with 10% FBS, 5 µg/ml transferrin, 10 ng/ml hEGF, 3 µg/ml insulin, 2 mM L-444 glutamine, 18 µg/ml hydrocortisone, 40 ng/ml dexamethasone, 5 ng/ml sodium selenite, 2% dimethyl sulfoxide (DMSO, cell culture grade), 100 U/ml penicillin, and 100 µg/ml streptomycin for 7 days before harvest. Where indicated, the infected cells were treated with lamivudine (3TC, 10 µM) to block de novo synthesis of HBV DNA replication.

2.7 PROMOTER REPORTER ASSAY

HepG2 cells were plated in a 96-well-plate and transfected with reporter plasmid ISG56-Luc plus control vector or vectors expressing the indicated gene of interest individually. Renilla luciferase reporter plasmid pRL-CMV was co-transfected to normalize the transfection efficiency across different transfected wells. Each transfection

received the same amount of total DNA (200 ng/well) with ISG56-Luc and gene of interest and pRL-CMV in 2:2:1 ratio. Recombinant IFN-α2a was purchased from PBL Biomedical Laboratories and the treatment was carried out at 1,000 IU/ml for 18 hours. Recombinant HBeAg was procured commercially (Virostat, product#8915). Furin inhibitor I was purchased from Calbiochem. Firefly luciferase and Renilla luciferase activities were measured by the dual luciferase assay kit (Promega) and BioTek Synergy 2 Multi-Mode Plate Reader.

2.8 CELLULAR mRNA qPCR

DNase I-treated total cellular RNA was used to generate cDNA by SuperScript III Reverse Transcriptase (Life Technologies). Real-time PCR was performed with SYBR Green Master (Roche) and the LightCycler 96 System (Roche) for detecting MxA, ISG56, OAS2, IRF9 and ISG20 mRNA by using gene-specific primers (Table A1). The relative expression levels of HBV RNA were normalized to β-actin from the same samples. The qPCR setting was 0.8 μM of primers, annealing and extension at 64°C for 45 cycles.

2.9 PRECORE mRNA qPCR

Total RNA and cccDNA purified from induced cell lines were subjected to qPCR specific for preCore mRNA, GAPDH, cccDNA, total Hirt HBV DNA and COX3 sequences (Table A1) were performed. LightCycler RNA master hydrolysis probes 2.7X kit (Cat# 04991885001, Roche) was used.

2.10 SUBCELLULAR FRACTIONATION

Cytoplasmic and nuclear fractionations were prepared by using the QIAgen Cell Compartment Kit (QIAgen #37502); microsomal fractionations were performed by using the BioVision Microsome Isolation Kit (BioVision cat# K249-50), per manufacturers' directions.

2.11 IMMUNOBLOTTING

Cells were lysed in 1× Laemmli buffer and denatured at 95°C for 10 min. Cell lysate was resolved in a 12% SDS-PAGE gel, and proteins were transferred onto an Immobilon PVDF-FL membrane (Millipore). The membranes were blocked with Western Breeze Blocker and probed with antibodies against aa 170-183 of precore/core (HBc170)(56), HA-tag (Sigma-Aldrich, cat# H3663; or Cell Signaling Technology, cat# 3724), FLAG-tag (Sigma-Aldrich, cat# F3165), Annexin I (Santa Cruz Biotechnology, cat# sc-65872), Lamin A/C (Santa Cruz Biotechnology, cat# sc-20681), PDI (Cell Signaling Technology, cat# 2446), β-actin (Santa Cruz Biotechnology, cat# sc-4778; or Millipore, cat# MAB1501), STAT1 (Cell Signaling Technology, cat# 9175), pSTAT1 (Tyr701) (Cell Signaling Technology, cat# 7649), and pSTAT2 (Cell Signaling Technology, cat# 4441). Bound antibodies were revealed by IRDye secondary antibodies and visualized using the LI-COR Odyssey system. Band intensity was measured by LI-COR Image Studio software.

2.12 CO-IMMUNOPRECIPITATION ASSAY

HepG2 or 293T cells were co-transfected with HA-p22, or HA-p22ΔCTD, or HA-VP24 and FLAG-Kα1 for 6 days. The harvested cells were lysed on ice with cell lysis

buffer containing 1% NP-40, 10 mM Tris.HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, protease inhibitor cocktail and Benzonase. After centrifugation to remove the cell debris, the clarified cell lysates were incubated with EZview Red Anti-HA Affinity Gel (Sigma-Aldrich) or Protein A/G beads pre-coated with anti-pSTAT1 or anti-pSTAT2 at 4°C overnight with gentle rotation. The beads were spun down the next day and resuspended gently with cold low salt wash buffer (50 mM Tris.HCl (pH 7.4), 5 mM EDTA, 150 mM NaCl, and Protease Inhibitors) for three times at 4°C followed by one time wash with high salt buffer (50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 500 mM NaCl, and Protease Inhibitors) and a desalting step with 20mM Tris HCL pH 7.4. The washed beads are re-suspended in Laemmli Buffer without DTT and spun down at high speed followed by collection of the supernatant and addition of DTT and immunoblotting technique is followed as described above.

2.13 IMMUNOFLUROSCENCE ASSAY

Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized by 0.5% Triton X-100 in PBS for 60 min at room temperature. Cells were subsequently blocked with blocking buffer (10% FBS plus 2% BSA in 1× PBS) for 60 min at room temperature, and then incubated with anti-HA (Cell Signaling Technology, cat#3724 or Sigma-Aldrich, cat#H3663) or anti-HBcAg (Dako, cat# B0586) diluted in the above-mentioned blocking buffer for overnight at 4ºC. After being washed with PBS, the cells were stained with Alexa Fluor 594 dye-conjugated secondary antibody (Life Technologies) and the nuclei were counterstained with DAPI for 60 min at room temperature. Finally, the cells were washed with PBS and subjected to Olympus FV1000 MPE confocal microscopy analysis with the

60× or 20× objective. Images were analyzed using the FV10-ASW 3.0 Viewer Software. The cytoplasmic HBV capsid particles were resolved in native agarose gel by electrophoresis and transferred onto nitrocellulose membrane, followed by capsid enzyme immunoassay assay (EIA) using antibodies against HBV core (DAKO, cat# B0586) as previously described(167, 168). The supernatant untagged HBeAg was detected by Chemiluminescence Immunoassay (CLIA) Kit (Autobio Diagnostics, cat# CL0312-2). The supernatant HA-HBeAg was detected by an in-house CLIA as previously described(156).

2.14 CHROMATIN IMMUNOPRECIPITATION ASSAY

ChIP-qPCR was performed on $2x10^6$ cells per reaction fixed with 1% formaldehyde using the ChIP-IT express kit with protein G magnetic beads (Active Motif) according to the manufacturer's protocol. Chromatin was sheared using EpiShear ultrasound sonicator (Active Motif) with delivery of 50-250 Joules depending on the cell line and precipitated with 5µg of specific or non-immune serum (NIS) control antibodies- anti-H3K27ac (abcam cat#ab4729), anti-IgG (Sigma cat#I8765-5MG) . After the removal of formaldehyde crosslinks, immunoprecipitated chromatin was deproteinized with proteinase K (10 µg/ml). The input control and pulled-down DNA fractions were purified with QIAquick PCR Purification Kit (Qiagen), served as templates in qPCR amplification using FastStart Essential DNA Probes Master kit (Roche). A serial dilution of pSP65 bearing 1 copy of HBV genome was used for quantification of FAM fluorescence, detected in LightCycler 480 System (Roche Diagnostics). Occupancy of the specific protein on cccDNA was expressed in percentage of input or in enrichment above NIS using -ΔΔCq method and further calculated as fold change with respect to the control.

2.15 ENZYME-LINKED IMMUNOASSAY

The cytoplasmic HBV capsid particles were resolved in native agarose gel by electrophoresis and transferred onto nitrocellulose membrane, followed by capsid EIA using antibodies against HBV core (DAKO, cat# B0586) as previously described(167, 168). The supernatant untagged HBeAg was detected by Chemiluminescence Immunoassay (CLIA) Kit (Autobio Diagnostics, cat# CL0312-2) or and the untagged HsAg was detected using the kit. The supernatant HA-HBeAg was detected by an in-house CLIA as previously described(156).

2.16 STATISTICAL ANALYSES

All data were analyzed by GraphPad Prism 5 and expressed as mean values ± standard deviation (SD) unless otherwise specified. Student t test was used to determine the statistical significance for in vitro experiments. $P<0.05$ was considered statistically significant.

2.17 PATIENTS

Between 2015 and 2016, patients with chronic HBV infection at Huashan Hospital of Fudan University (Shanghai, China) were evaluated. Individuals with concurrent hepatitis C, hepatitis D, HIV, autoimmune liver disease, or alcoholic liver disease were excluded. Twenty-one treatment-naïve patients (11 HBeAg(+) and 10 HBeAg(-)) with liver biopsies performed at baseline were enrolled. HBV sequence analysis revealed that all the HBeAg(-) patients were infected with precore stop codon mutation (G1896A) virus. Both HBeAg(+) and HBeAg(-) patients received IFN-α monotherapy and a second liver

biopsy was performed at 24 weeks. In the present study, the ULN for ALT was set at 50 U L-1. Patient characteristics are summarized in Table 2. All human subjects were recruited upon obtaining informed written consent. The study was approved by the Institutional Ethics Committee for human studies at Huashan Hospital (IRB# 2016-123 and 2018-131).

2.18 LIBRARY CONSTRUCTION FOR RNA-SEQ, SEQUENCING PROCEDURES, AND DATA ANALYSIS

This was provided by our collaborators from Fudan University, Dr. Jiming Zhang. Total RNA of liver biopsies were extracted by using RNeasy mini kit (Qiagen). Strand-specific libraries were prepared using the TruSeq® Stranded Total RNA Sample Preparation kit (Illumina) following the manufacturer's instructions. Briefly, ribosomal RNA was removed from total RNA by Ribo-Zero rRNA removal beads. Following purification, the mRNA was fragmented into small pieces using divalent cations under 94°C for 8 min. The RNA fragments were copied into the first strand cDNA by using reverse transcriptase and random primers, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. These cDNA fragments then went through an end repair process, the addition of a single 'A' base, and then ligation of the adapters. The products were then purified and enriched with PCR to create the final cDNA library. Purified libraries were quantified by Qubit® 2.0 Fluorometer (Life Technologies) and validated by Agilent 2100 bioanalyzer to confirm the insert size and calculate the mole concentration. Cluster was generated by cBot with the library diluted to 10 pM and then were sequenced on the Illumina HiSeq 2500. The library construction and sequencing were performed at Shanghai Biotechnology Corporation.

Sequencing raw reads were preprocessed by filtering out rRNA reads, sequencing adapters, short-fragment reads and other low-quality reads. Tophat v2.1.0 was used to map the cleaned reads to the human h19 reference genome with two mismatches(169). After genome mapping, Cufflinks v2.1.1 was run with a reference annotation to generate FPKM values for known gene models, and the differentially expressed genes (DEGs) were identified using Cuffdiff(170). The p-value significance threshold in multiple tests was set by the false discovery rate (FDR)(171). The fold-changes were also estimated according to the FPKM in each sample. The differentially expressed ISG genes were selected using the following filter criteria: $p \leq 0.05$ 554 and fold-change ≥ 2.

Table 2: Baseline Characteristics of Enrolled Patients

	HBeAg(+) (n=11)	HBeAg(-) (n=10)	p-value
Male gender (n, %)	9 (81.8%)	8 (80.0%)	1.000
Age (year)	30 (16, 44)	30.5 (25, 57)	0.306
HBsAg (log10, IU/ml)	4.39 (3.50, 4.72)	3.23 (1.50, 4.27)	0.001
HBeAg (s/co)	831.42 (3.75, 1565.70)	0.37 (0.34, 0.84)	<0.001
HBV DNA (log10, IU/ml)	7.30 (2.70, 7.70)	5.58 (2.70, 7.05)	0.024
ALT (IU/L)	178 (56, 325)	100 (41, 474)	0.067

AST (IU/L)	59 (46, 147)	58.5 (28, 250)	0.290
TB (µmol/L)	12.0 (7.0, 22.0)	11.6 (9.0, 23.4)	0.778

CHAPTER 3

RESULTS

3.1 HBV PRECORE PROTEIN EXISTS IN A p22 FORM IN CELL CULTURES

To validate the presence of p22 during HBV replication and HBeAg production, the plasmid pHBV1.3 that expresses all HBV proteins was transfected into HepG2 cells. The expression of intracellular core protein (p21) and p22 was detected by western blot using antibodies against the last 14 amino acids (aa) of precore/core CTD (52, 53) (Figure 5, lane 1). The expression of core protein (p21) and precore-derived p22 from pHBV1.3 was further confirmed by transfection of the core-null HBV plasmid pHBV1.3ΔC (Figure 5, lane 2), and by transfection of core-expressing plasmid pHBc alone (Figure 5, lane 3) and precore-expressing plasmid pcHBe alone (Figure 5, lane 4). The protein size of p22 intermediate was further confirmed by expression of the precore ORF without the 19 aa signal peptide coding sequence, which was predicted to express a 22kD protein (Figure 5, lanes 4 and 5). Transfection of pcHA-HBe produced an intracellular N-terminally HA-tagged precore protein of 24KD (HA-p22) (Figure 5, lane 6). However, the secreted HA-HBeAg (HA-p17) was not detected intracellularly, which is consistent with previous studies demonstrating that the HBeAg/p17 is rapidly secreted out of the cell from Golgi after furin cleavage and does not retain in the ER-Golgi (18, 52). Collectively, the results demonstrate that the intracellular HBV precore protein predominantly exists as the p22 form.

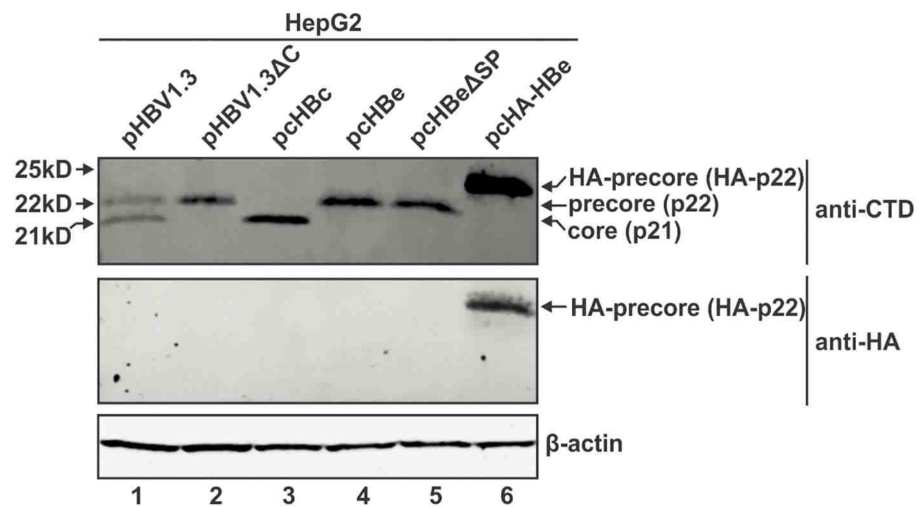


Figure 5: HBV precore protein exists as a p22 form intracellularly

HepG2 cells were seeded in 12-well plate and mock transfected or transfected with pHBV1.3, pHBV1.3ΔC, pcHBc, pcHBe, pcHBeΔSP and pcHA-HBe, as indicated. Cells were harvested at day 5 post-transfection, and the proteins were analyzed by Western blot using antibodies against CTD and HA-tag. β-actin served as loading control

3.2 SUBCELLULAR DISTRIBUTION OF p22

Next, we examined the distribution of p22 amongst different cellular compartments. Using HepHBe4 cell line that constitutively expresses HA-tagged p22 (HA-p22) and HBeAg (HA-HBeAg), and the tetracycline-inducible HepBHAe82 cells that express HA-p22 and HA-HBeAg in a cccDNA-dependent manner (52). The immunofluorescence microscopy demonstrated that while HA-p22 is predominantly found in the cytoplasm, it is also able to translocate to the nucleus (Figure 6A). This was further validated with subcellular fractionation of HA-p22 expressing plasmid transfected-HepG2 cells showing the presence of both the cytoplasmic and nuclear forms of p22 (Figure 6B). Since p22 is translated in the ER lumen and have been suggested to partly retro-translocate to cytosol through the ER-associated degradation pathway (14, 55), we did a microsomal fractionation of cells transfected with HA-p22 and found that p22 exists in both the microsomal and cytosolic compartments (Figure 6C, lane 1). Furthermore, when cells were treated with the furin inhibitor I to block furin-mediated cleavage of p22 and subsequent release of HBeAg from Golgi, the p22 was seen to significantly accumulate in the microsomal fraction without further increase in the cytosolic compartment (Figure 6C, lane 2 vs lane 1), indicating that, once p22 reaches Golgi complex, it cannot be transported back to the cytosol. The mechanism underlying the sorting of p22 into Golgi for HBeAg process and ER-to-cytosol retro-translocation awaits further investigation. Garcia et al had previously shown that that 70-80% of this signal peptidase-cleaved product was localized on the cytoplasmic side of the microsomal vesicles and was not associated with the membranes and only the remaining (190).

20-30% appeared to be completely translocated into the lumen of the microsomes. The group also showed that a deletion mutant lacking the carboxy-terminal nucleic acid binding domain of the precore protein was similarly partitioned between the lumen of the microsomes and the cytoplasmic compartment, indicating that this highly charged domain is not responsible for the aborted translocation.

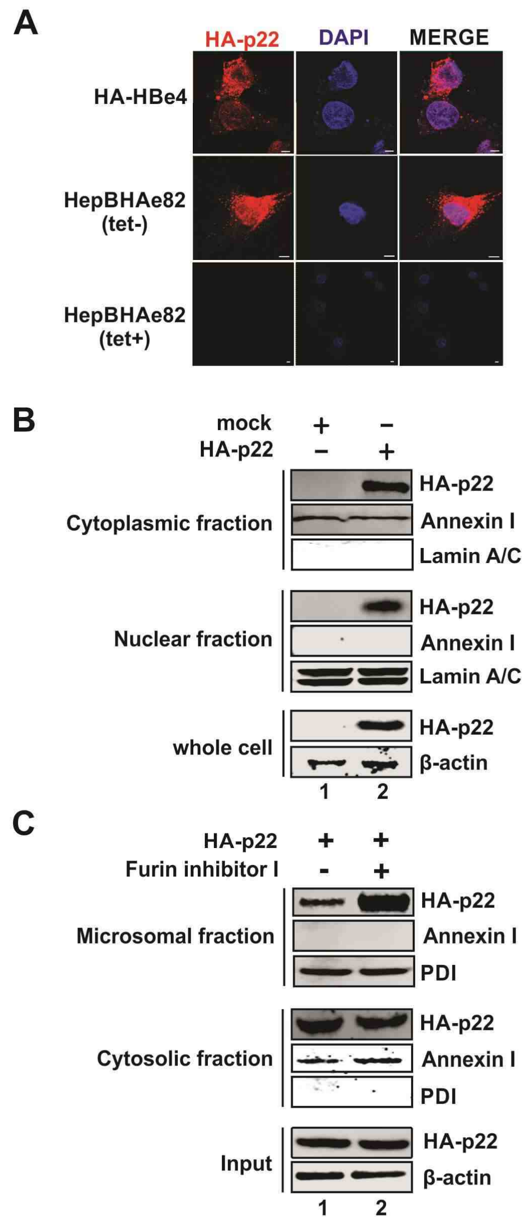


Figure 6: The subcellular localization of p22

(A) HepHA-HBe4 cells and non-induced (tet+) and induced (tet-, day 14) HepBHAe82 cells were subjected to immunofluorescence microscopy for HA-p22 (red) staining. The cell nuclei were stained by DAPI (blue). (B) HepG2 cells in 12-well plate were transfected

with either control vector or pcHA-HBe (HA-p22). Cells were harvested at day 5 post-transfection and subcellular fractionation was carried out to detect HA-p22 in cytoplasmic and nuclear lysates by Western blot using anti-HA antibody. Annexin I and Lamin A/C served as a marker and loading control for the cytoplasmic and the nuclear fractions, respectively, and β-actin served as loading control for whole cell lysate. (C) 293T cells in 12-well plate were transfected with HA-p22 and left untreated or treated with Furin Inhibitor I (1μM). Cells were harvested at day 5 post-transfection and subjected to microsomal fractionation analysis. Membrane-associated HA-p22 and cytosolic HA-p22 were detected by Western blot using anti-HA antibody. Annexin I and PDI served as a marker and loading control for the cytosolic and the membrane fractions, respectively, and β-actin served as loading control for whole cell lysate.

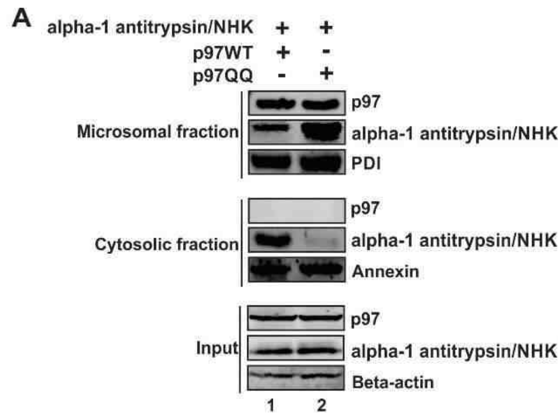
3.3 A PORTION OF p22 IS RELEASED BACK INTO THE CYTOSOL THROUGH AN ERAD-INDEPENDENT MECHANISM

From our previous figure (Figure 6 A and B), p22 is shown to be present in both the cytoplasmic and the nuclear compartments. Previously, it has been suggested that after the cleavage of the signal peptide and formation of p22 protein, the protein uses the Endoplasmic Reticulum-Associated Degradation (ERAD) pathway to retro-translocate to the cytosol. All misfolded proteins retro-translocating from the ER lumen to the cytosol use p97 or VCP channel, the ATP-using transporter allowing translocation of protein to the cytosol. Due to the reduced lysine content on the protein, the p22 was expected to escape ubiquitination and hence, proteasome-mediated degradation. Thus, in order to study route utilized by p22 to translocate from ER to the cytosol, we over-expressed the wild type or dominant-negative mutant (unable to use ATP) of the p97 translocon in 293T cells along with a positive control protein, alpha-1 antitrypsin (a misfolded protein known to use the ERAD pathway for retro-translocation) and our HA-tagged protein of interest, HA-p22. 293 T cells allowed a higher expression levels of the above-mentioned proteins and hence, provided a clearer data for the fractionation study. The transfected cells were collected 5 days post transfection and subjected to microsomal fraction. As seen in the Figure 7A, p97 is seen only in the microsomal fraction and alpha-1 antitrypsin fails to translocate to the cytosol in presence of p97-mutant (middle panel, lane 2) compared to p97-wild type (middle panel, lane 1) and in the case of p97-mutant, p22 is seen to accumulate in the microsomal fraction (top panel, lane 2). The over-expression of p97 doesn't affect the distribution of p22 between the microsomal compartments as seen in Figure 6B, lanes 1 and 4. The reduced HA-p22 seen for the p97 mutant is probably the result of the reduced

expression of HA-p22 on expression of the p97 mutant plasmid as seen in the input (Figure 7B, bottom panel, lanes 3 and 6). The p97 mutant fails to inhibit the retro-translocation of p22 (Figure 7B middle panel, lanes 1-3) unlike in the case of alpha-1 antitrypsin. Further, in order to induce more p22 to retro-translocate to cytosol, we treated the HA-p22 and p97 (wildtype and mutant) transfected cells with furin inhibitor (Figure 7B, lanes 4-6. However, the prevention of formation of HBeAg didn't allow for a larger amount of p22 retro-translocating to the cytosol (Figure 7B, middle panel, lanes 3-6). In fact, as shown in Figure 6C, most of the newly-formed p22 prefers to go through the secretion pathway to form HBeAg and any treatment with furin treatment causes an accumulation of the p22 in the microsomal fraction (probable, golgi, mostly). Thus, the results suggest the use of an ERAD-independent mechanism by a portion of p22 to retro-translocate to cytosol after formation.

Short peptides called cell-penetrating peptides (CPPs) have been shown to be able to transport cargo across intracellular membranes through various mechanisms, such as, direct penetration or endocytosis-mediated translocation. The trans-activating transcriptional activator (TAT) from HIV-1 could be efficiently taken up from the surrounding media by numerous cell types in culture and Human Papillomavirus use CPPs to penetrate the intracellular membrane in order to trigger retrograde trafficking of the viral unit to the nucleus (191). The CTD of p22 has various properties pertaining to CPP, such as, presence of positively-charged peptides, hydrophobic amino acids etc. Thus, we hypothesized that p22 is able to retro-translocated into the cytosol through the use of its CTD as a CPP and penetrating the ER membrane to get across. To test this, we designed a mutant p22 with a CTD-deletion. HepG2 cells transfected with HA-p22 or p22ΔCTD were

subjected to microsomal fractionation 5 days post-transfection. However, both the wild type p22 and the p22ΔCTD mutant were observed in the microsomal fraction suggesting the absence of CPP-dependent mechanism for the retro-translocation.



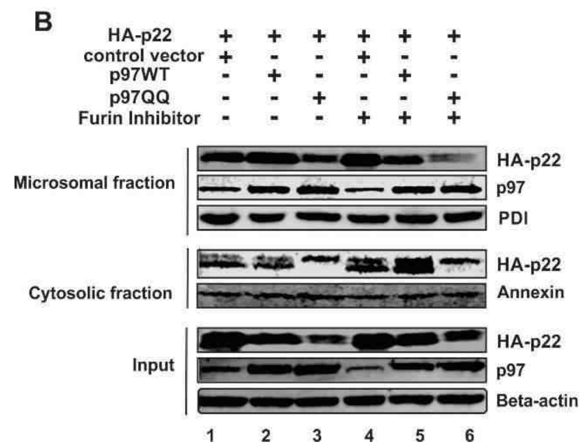


Figure 7: p22 doesn't use ERAD pathway for retro-translocation to cytosol from the ER lumen

(A) 293T cells in a 12-well plate were transfected with alpha-1 antitrypsin. Cells were harvested at day 5 post-transfection and subjected to microsomal fractionation analysis. Inputs of alpha-1 antitrypsin, membrane-associated alpha-1 antitrypsin and cytosolic alpha-1 antitrypsin were detected by Western blot using anti- alpha-1 antitrypsin antibody. (B) 293T cells in a 12-well plate were transfected with HA-p22 or control vector and left

untreated or treated with Furin Inhibitor I (1μM). Cells were harvested at day 5 post-transfection and subjected to microsomal fractionation analysis. Input hA-p22, membrane-associated HA-p22 and cytosolic HA-p22 were detected by Western blot using anti-HA antibody. (A & B) Annexin I and PDI served as markers and loading controls for the cytosolic and the membrane fractions, respectively, and β-actin served as loading control for whole cell lysate.

3.4 p22 DOES NOT FORM CAPSID OR SUPPORT DNA REPLICATION

HBV core protein and p22 share the exact same amino acid sequence except for the 10 amino acid N-terminal extension in the p22 protein as shown in Figure 1A. While core protein can form dimers through an inter-disulfide bond formation between their Cys 61 and these dimers in-turn lead to the formation of a capsid, *Schodel et al* had shown that the bacterially expressed HBeAg (p17) failed to form a capsid in E. coli like core. However, mutation of the cysteine at -7 aa position in the precore domain to a glutamine (C-7Q) allowed capsid formation (54). In the present study, we tested the ability of full length precore/p22 to form a capsid in the mammalian cells. As shown in Figure 8A, comparing to the replication-competent plasmid pHBV1.3, the core-null pHBV1.3ΔC failed to support HBV pgRNA encapsidation and DNA replication due to the defect of capsid formation, though p22 was expressed (Figure 8A, lane 2 vs lane 1). The viral replication of pHBV1.3ΔC was rescued by transcomplementing core but not p22 or HA-tagged p22 (Fig. 8A, lanes 3-5). Furthermore, the p22 with C-7Q mutation remains defective in capsid formation (Figure 8B). Thus, p22 fails to form a capsid unlike core, suggesting that the N-terminal 10 aa precore domain of p22 negatively regulates capsid assembly, and p22 does not play an essential role in HBV DNA replication.

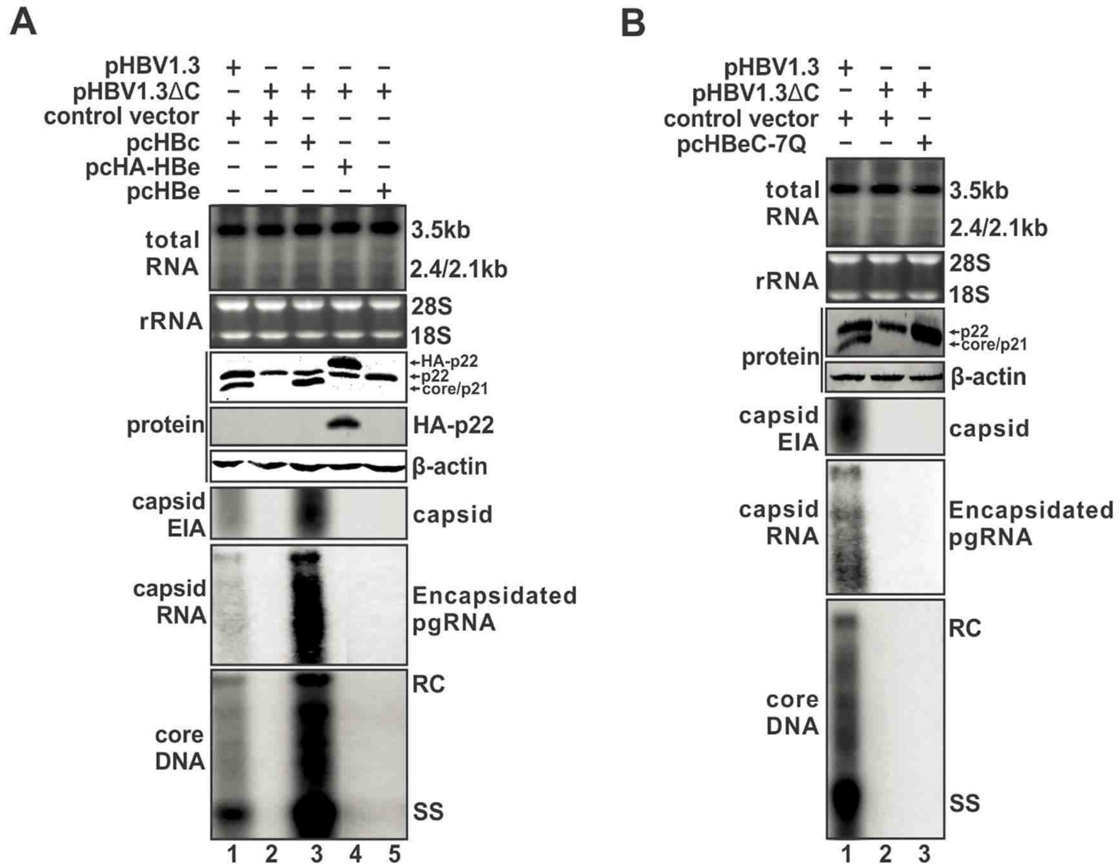


Figure 8: p22 fails to support HBV capsid formation and DNA replication

(A) HepG2 cells in a 12-well plate were co-transfected with either pHBV1.3 and control vector, or pHBV1.3ΔC and control vector or pcHBc, pcHBe or pcHA-HBe, as indicated. Cells were harvested at day 5 post-transfection, viral total RNA and encapsidated pgRNA were analyzed by Northern blot; core DNA was detected by Southern blot; cytoplasmic capsid was analyzed by native capsid gel EIA assay. The expression of core and precore protein was detected by Western blot using core antibody against the CTD domain, and the HA-tagged p22 was detected by Western blot using anti-HA antibody. β-actin served as loading control. For RNA analysis, each lane was loaded with 10 μg of total RNA and probed with a genome-length, plus-strand-specific HBV riboprobe. Ribosomal RNAs (28S

and 18S) are presented as loading controls. The positions of HBV pgRNA (3.5kb) and subgenomic surface RNAs (2.4kb and 2.1kb) are indicated. HBV core DNA was probed with genome-length, minus-strand-specific HBV riboprobe. The positions of relaxed circular (RC) and single-stranded (SS) DNAs are indicated. (B) HepG2 cells in a 12-well plate were co-transfected with either pHBV1.3 and control vector, or pHBV1.3ΔC and control vector or pcHBeC-7Q, as indicated, for 5 days. The analyses of viral RNA, DNA, and proteins were performed in the same way as described above.

3.5 FUNCTIONS OF CYTOPLASMIC p22

3.5.1. p22 INHIBITS ISRE PROMOTER ACTIVITY AND ISG EXPRESSION

It has been reported that HBeAg(-) patients respond better to IFNα therapy than HBeAg(+) patients (47-51), but the underlying mechanisms remain elusive. In this study, consecutive liver biopsies from 11 HBeAg(+) and 10 HBeAg(-) patients at baseline and after 24-week IFN-α monotherapy were collected for RNA-seq analysis with special focus on ISG induction profile. As shown in Figure 9, across a group of 28 ISGs with more than 2-fold induction in each patients at the end of IFN-α therapy, HBeAg(+) patients have an overall weaker ISG induction compared to HBeAg(-) patients, indicating that the circulating HBeAg and/or intracellular p22 might have a negative effect on the IFN-signaling pathway. This experiment was done by Dr. Jiming Zhang's Lab at Fudan University and they were kind enough to share the data with us.

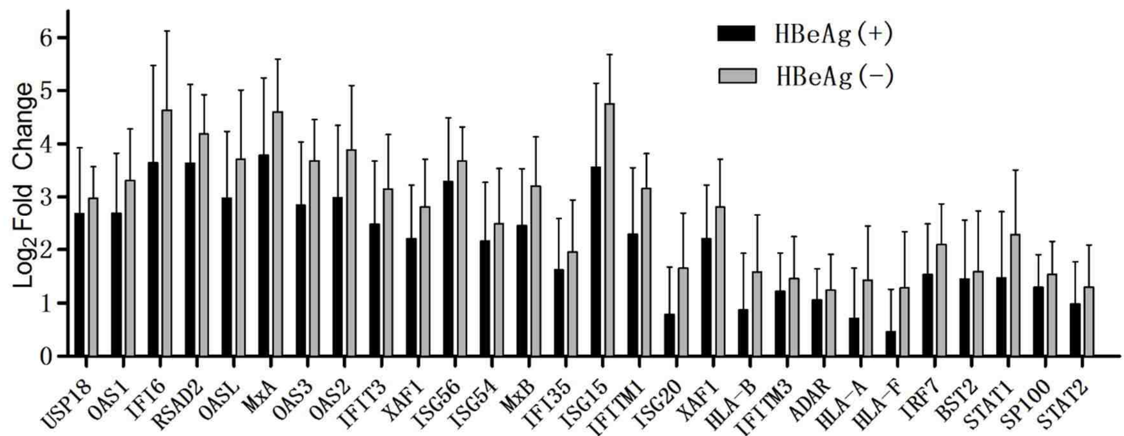


Figure 9: The induction of intrahepatic ISGs in HBeAg(+) and HBeAg(-) chronic Hepatitis B patients under IFN-α treatment

Liver biopsies were performed from 11 HBeAg(+) and 10 HBeAg(-) patients at baseline and after 24-week IFN-α monotherapy, respectively. Total cellular mRNA samples were subjected to RNA sequencing and the expression abundance of genes was collected. The fold change of the expression abundance for 28 ISGs (filter criteria: $p \leq 0.05$ and fold-change ≥ 2) in HBeAg(+) and HBeAg(-) groups was plotted. Each histogram represents the ratio of FPKM for each gene between baseline and after therapy, whose values were then logarithmically transformed.

In order to investigate the effect of p22 and HBeAg on interferon-sensitive response element (ISRE) activity, we co-transfected HepG2 cells with ISG56 promoter-controlling luciferase reporter plasmid, ISG56-Luc, along with control vector or different HBV protein-expressing plasmids, followed by IFN-α treatment. As shown in Figure 10A, while the control group exhibited a high ISG56 promoter activity upon IFN-α stimulation, expression of HBV core or p22 (wildtype or HA-tagged) reduced this activity significantly, confirming an inhibitory effect of HBV p22 or its extracellular product, HBeAg, on IFN signaling. No such effect was seen when other HBV proteins, such as HBx, HBV surface proteins, or HBV polymerase, were co-expressed, suggesting that the inhibitory effect on ISG56 promoter activity is specific to p22 and core proteins. In order to further identify whether the inhibition was mediated by intracellular p22 or extracellular HBeAg, ISG56-Luc-transfected cells were treated with either recombinant HBeAg which did not show any inhibition of IFN-α activity or with furin inhibitor I, which prevents the secretion of HBeAg in p22 transfected cells, did not attenuate the p22-mediated reduction of ISG56 promoter activity, suggesting that the intracellular p22, but not the secreted HBeAg, blocks IFN-α signaling. It is worth noting that blocking HBeAg secretion by furin inhibitor did not further inhibit ISG56-Luc activity, which is consistent with the fact that the furin inhibitor-arrested intracellular p22 does not retro-translocate to cytosol (Figure 6C and 7B), where the IFN signaling takes place. Furthermore, the effect of HBV core and p22 on ISG mRNA levels upon IFN-α treatment was examined. Quantitative PCR analysis of the relative expression of ISG56 and MxA demonstrated that HBV p22 and core protein markedly reduced the ISG transcripts induced by IFN-α (Figure 10B), further confirming an inhibitory effect of these HBV proteins on the IFN-elicited ISG production.

Figure 10: Intracellular p22 suppresses IFN-α signaling

(A) HepG2 cells were seeded in 96-well-plate and co-transfected with ISG56-Luc and control vector or other HBV protein- expressing plasmids, as indicated, together with pRL-CMV. 5 days later, the transfected cells were left untreated or treated with human IFN-α (1,000 IU/ml) for 18 h. Where indicated, exogenous treatment with recombinant HBeAg

(rHBeAg, 50ng/ml) or treatment with furin inhibitor I (1 μM) was carried out. Firefly and *Renilla* luciferase activities were measured where the latter served as the internal control to normalize transfection efficiency. The relative luciferase activities were plotted as fold changes versus the control group without IFN-α treatment (mean±SD, n=4). (B) HepG2 cells in 12-well-plate were transfected with control vector, pcHBc (core/p21) or pcHBe (p22) as indicated. At day 5 post-transfection, the transfected cells were treated with human IFN-α (1,000 IU/ml) for 30 min and total cellular RNA was extracted and subjected to MxA and ISG56 mRNA qPCR analysis. The relative individual gene induction was plotted as fold changes versus the control group (mean±SD, n=4).

3.5.2 p22 INHIBITS ISG PRODUCTION IN HBV INFECTION SYSTEM *in vitro*

In order to validate the above seen effects of p22 in a more physiological setting, we resorted to the infection model using HepG2-NTCP12 cells. We made two types of p22-null viruses, one contains a start codon mutation of the precore ORF in viral genome, and the second one harbors the precore G1896A stop codon mutation. The wildtype and p22-null virus were used to infect the HepG2-NTCP cells with same inoculum size for 7 days, 3TC was included during the infection to prevent de novo HBV DNA replication and the intracellular cccDNA amplification. Core immunofluorescence demonstrated that both wildtype and p22-null viruses successfully established the infection (Figure 11 A and D). The true precore-null nature of the mutant viruses was confirmed by HBeAg and HA-HBeAg CLIA (Figure 11 B and E). The infected cells were treated with IFN-α and the expression of several ISGs were analyzed by qPCR. A stronger induction of analyzed ISGs was seen in cells infected with p22-null HBV compared to those infected with wildtype virus (Figure 11 C and F). A similar infection experiment was carried out with the PXB cells where the wildtype and p22-null (∆e) viruses were used to infect the PXB cells with same inoculum size for 7 days, 3TC was included during the infection to prevent de novo HBV DNA replication and the intracellular cccDNA amplification. The infected cells were treated with IFN-α and the expression of several ISGs were analyzed by qPCR. A stronger induction of analyzed ISGs was seen in cells infected with ∆e HBV compared to those infected with wildtype virus (Figure 11 G). The above results from HBV infection system further validated the inhibitory effect of p22 on IFN-α signaling and ruled out the possibility of the effects being an effect from overexpression of the protein.

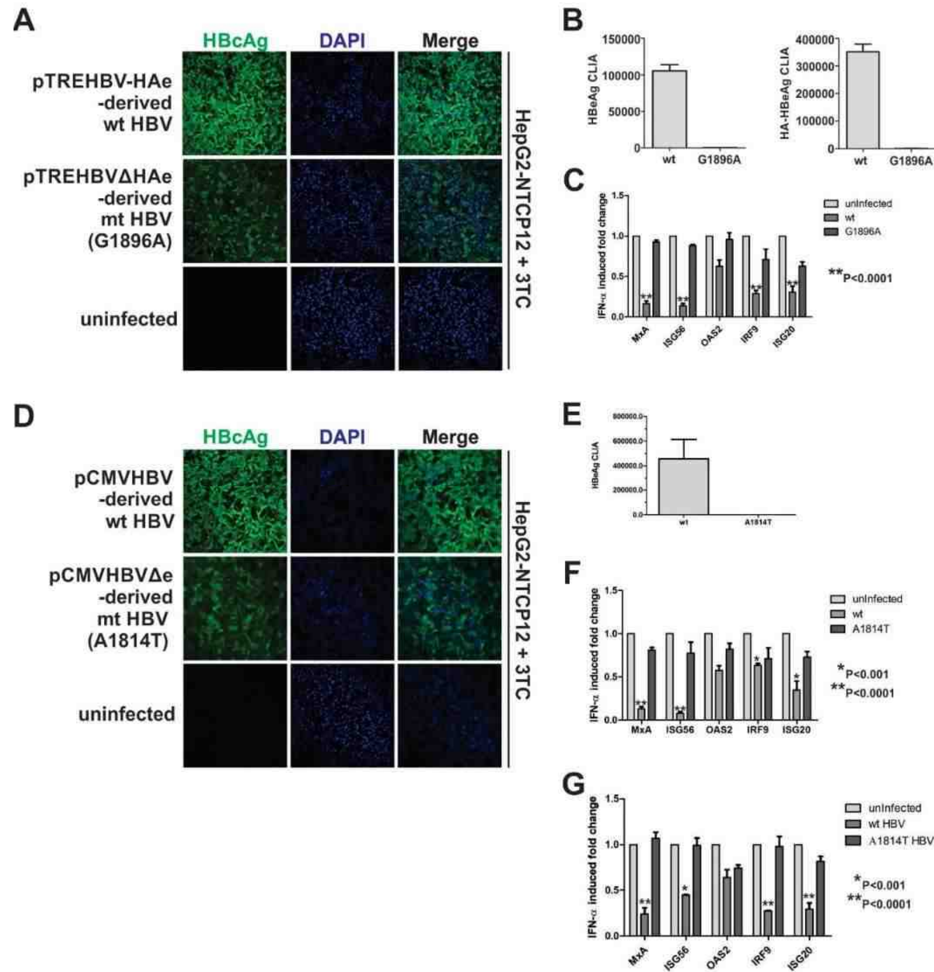


Figure 11: p22 downregulates IFN-α-mediated ISG induction in HBV infection system *in vitro*

HepG2-NTCP12 cells in a 96-well plate were left uninfected or were infected with the wildtype HBV, G1896A mutant A1814T precore-null viruses, respectively, at 500 VGE/cell for 24 hours. The infected cells were then cultured in the presence of 10 µM of 3TC for 7 days. HBV infectivity was assessed by HBcAg immunofluorescence (A and D). The precore-null nature of the mutant viruses was confirmed with supernatant HBeAg and HA-HBeAg CLIA (B and E). For another set of cells uninfected or infected with wildtype or mutant viruses in the same way as described above, the cells were treated with IFN-α

(1,000 IU/ml) for 30 min prior to cell harvest, and the induced expression of MxA, ISG56, OAS2, IRF9 and ISG20 was measured by qPCR analysis. The relative individual gene induction by IFN-α was plotted as fold changes versus the uninfected control group (mean±SD, n=3) for HepG2-NTCP12 cell infection (C and F) and PXB cell infection (G).

3.5.3 p22 INHIBITS THE NUCLEAR TRANSLOCATION OF pSTAT1/2 THROUGH COMPETING THEIR INTERACTION WITH KARYOPHERIN

It is well known that IFN-α induces ISG production through the canonical JAK-STAT pathway, leading to the nuclear translocation of phosphorylated STAT1/2 heterodimer in the ISGF3 complex and binding with ISG promoters containing ISRE element (56) (Figure 12A). In order to elucidate the mechanism of p22-mediated inhibition of IFN-signaling, we assessed the effect of p22 on STAT phosphorylation upon IFN-α stimulation. As shown in Figure 12B, the levels of pSTAT1 and pSTAT2 in whole cell lysates of HepG2 cells activated by IFN-α were unchanged in the presence of p22 compared to control group, suggesting a proper binding of IFN to its receptors and phosphorylation of the STAT1/2. However, subcellular fractionation analysis showed that p22 led to an accumulation of the pSTAT1 and pSTAT2 in cytoplasm corresponding to a reduced pSTAT1/2 in the nucleus, suggesting that p22 blocks the nuclear translocation of pSTAT1/2 complex (Figure 12C). Interestingly, HBV core protein did not prevent the nuclear importation of pSTAT1 and pSTAT2 (Figure 12D), indicating a different mode of inhibition of the IFN-signaling by the core protein, which awaits further investigation.

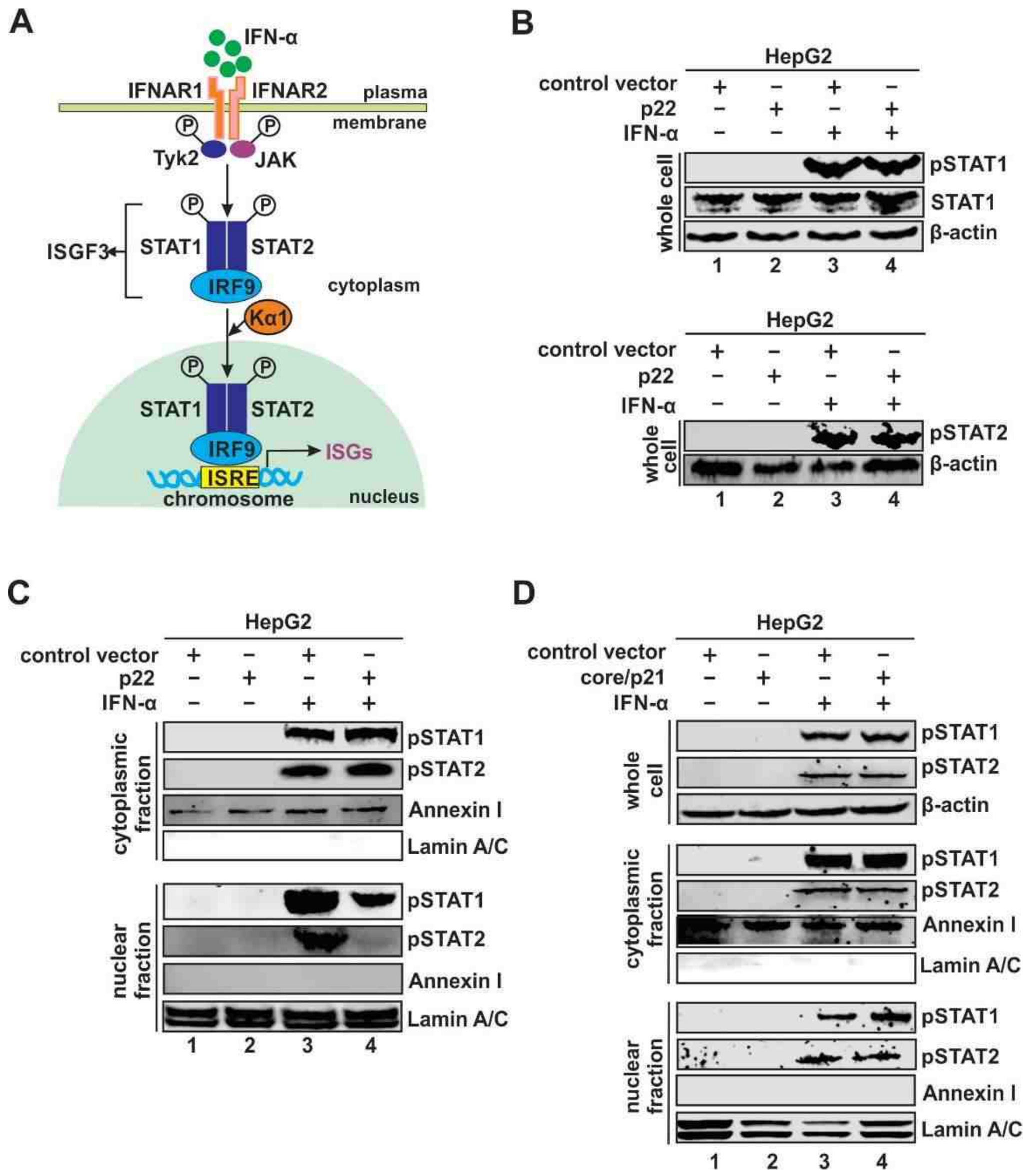


Figure 12: p22 blocks the nuclear translocation of STAT1/2

(A) Schematic illustration of IFN-α-elicited JAK-STAT signaling and the activation of ISG expression. IFN-α binds to its cognate receptor IFNAR1 and IFNAR2 on the cell membrane and activates the JAK-STAT pathway. The phosphorylated STAT1 and STAT2 form heterodimer, followed by interaction with IRF9 to form ISGF3. Kα1, a nuclear transporter protein binding ISGF3, is essential to mediate the nuclear import of ISGF3. The

ISGF3 binds to ISRE in DNA to activate transcription of ISGs. (B) HepG2 cells in 12-well plate were transfected with either control vector or pcHBe (p22). At day 5 post-transfection, the transfected cells were left untreated or treated with IFN-α (1,000 IU/ml) for 30 min. Total STAT1, pSTAT1 and pSTAT2 were detected by Western blot, with β-actin serving as a loading control. (C) HepG2 cells in 6-well-plate were transfected with control vector or p22 for 5 days, followed by mock treatment or IFN-α treatment (1,000 IU/ml) for 30 min. The harvested cells were subjected to cell fractionation, and the levels of the pSTATs were detected in cytoplasmic (top panel) and nuclear (bottom panel) fractions. Annexin I and Lamin A/C served as a marker and loading control for the cytoplasmic and nuclear fraction, respectively. (D) HepG2 cells in 6-well-plate were transfected with control vector or core/p21. At day 5 post-transfection, the cells were left untreated or treated with IFN-α (1,000 IU/ml) for 30 min. Cell fractionation and pSTAT1 detection in cytoplasmic and nuclear fractions were conducted in the same way as described above.

In order to further study the mechanism underlying p22-mediated blockage of nuclear translocation of ISGF3 complex, we looked at the protein-protein interaction between p22 and nuclear transporter, Kα1. The ISGF3 complex requires the interaction with Kα1 for its nuclear entry and activation of ISG expression. Hence, we performed a co-immunoprecipitation experiment with HA-tagged p22 and FLAG-tagged Kα1 overexpression in 293T cells. 293T cells supported a higher expression levels of the above-mentioned proteins and hence, provided a clearer data for the immunoprecipitation study. Immunoprecipitation with anti-HA antibody and detection of Kα1 through anti-FLAG antibody confirmed p22's ability to interact with Kα1 (Figure 13A). The HA-tagged Ebola VP24 has been shown to interact with Kα1 and block the nuclear translocation of pSTAT1 and thus served as a positive control (57). Since both p22 and ISGF3 complex bind to Kα1 to enter the nucleus, we, thus, hypothesized that p22 might be competing with ISGF3 complex to bind to Kα1 and preventing the entry of the ISGF3 into the nucleus. Hence, we performed a competition assay where we treated cells with IFN-α in the absence or presence of p22. Immunoprecipitation with anti-pSTAT1 antibody followed by detection of FLAG-Kα1 demonstrated a reduced amount of Kα1 binding to pSTAT1 in the presence of p22 (Figure 13B). Thus, we conclude that p22 competes with pSTAT1 to bind to the nuclear transporter Kα1, preventing the entry of pSTAT1 into the nucleus.

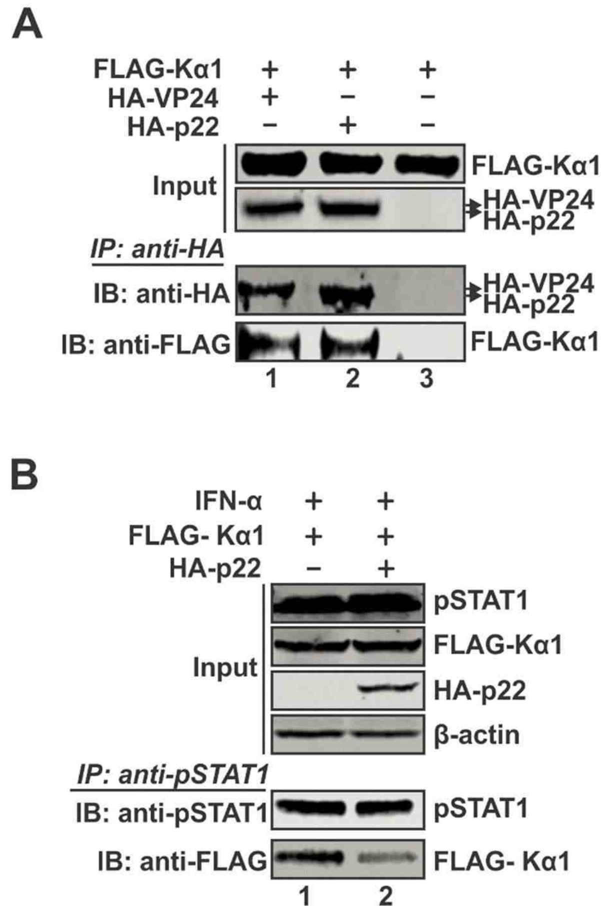


Figure 13: p22 competes with pSTAT1 for binding to Kα1

(A) 293T cells in 6-well plate were co-transfected with FLAG-Kα1 and HA-VP24, or HA-p22, or control vector as indicated. 5 days later, the transfected cells were lysed and subjected to co-immunoprecipitation with beads coated by anti-HA antibody and the presence of co-precipitated proteins was analyzed by Western blot using anti-HA and anti-FLAG antibodies. (B) 293T cells in 6-well-plate were co-transfected with FLAG-Kα1 plus control vector or HA-p22 for 5 days, followed by mock treatment or IFN-α treatment (1,000 IU/ml) for 30 min. The cells were then subjected to co-immunoprecipitation with

anti-pSTAT1-conjugated beads, and the presence of pulled-down FLAG-Kα1 was analyzed by Western blot using anti-FLAG antibody.

3.5.4 p22 BLOCKS IFN-α SIGNALING THROUGH THE CTD DOMAIN

HBV core protein possesses a bipartite nuclear localization signal in its arginine-rich C-terminal domain (CTD) (16). Since p22 shares the same C-terminus with core, we hypothesized that the CTD might signal p22 for its nuclear translocation and hence, might be responsible for competing with the ISGF3 complex to bind to Kα1. We created a CTD-deletion mutant of HA-p22 (HA-p22ΔCTD) and this mutant showed its inability to translocate to the nucleus from cytoplasm (Figure 14 A) when overexpressed in HepG2 cells followed by a subcellular fractionation. We have also examined the mutant's ability to interact with Kα1 through co-immunoprecipitation where FLAG-tagged Kα1 was over-expressed alone or co-expressed with either HA-p22 or HA-p22ΔCTD in 293T cells. 293T cells supported a higher expression levels of the above-mentioned proteins and hence, provided a clearer data for the immunoprecipitation study. While HA-p22 was seen to interact with Kα1, HA-p22ΔCTD mutant did not co-precipitate with Kα1, suggesting the role of p22's CTD in its interaction with the nuclear transporter and in nuclear translocation of p22 (Figure 14B). Moreover, ISG56-Luc reporter assay demonstrated that the HA-p22ΔCTD mutant failed to inhibit IFN-α-induced ISG56 promoter activity (Figure 14C). Thus, the CTD domain of p22 is required for the inhibition of IFN-induced JAK-STAT signaling.

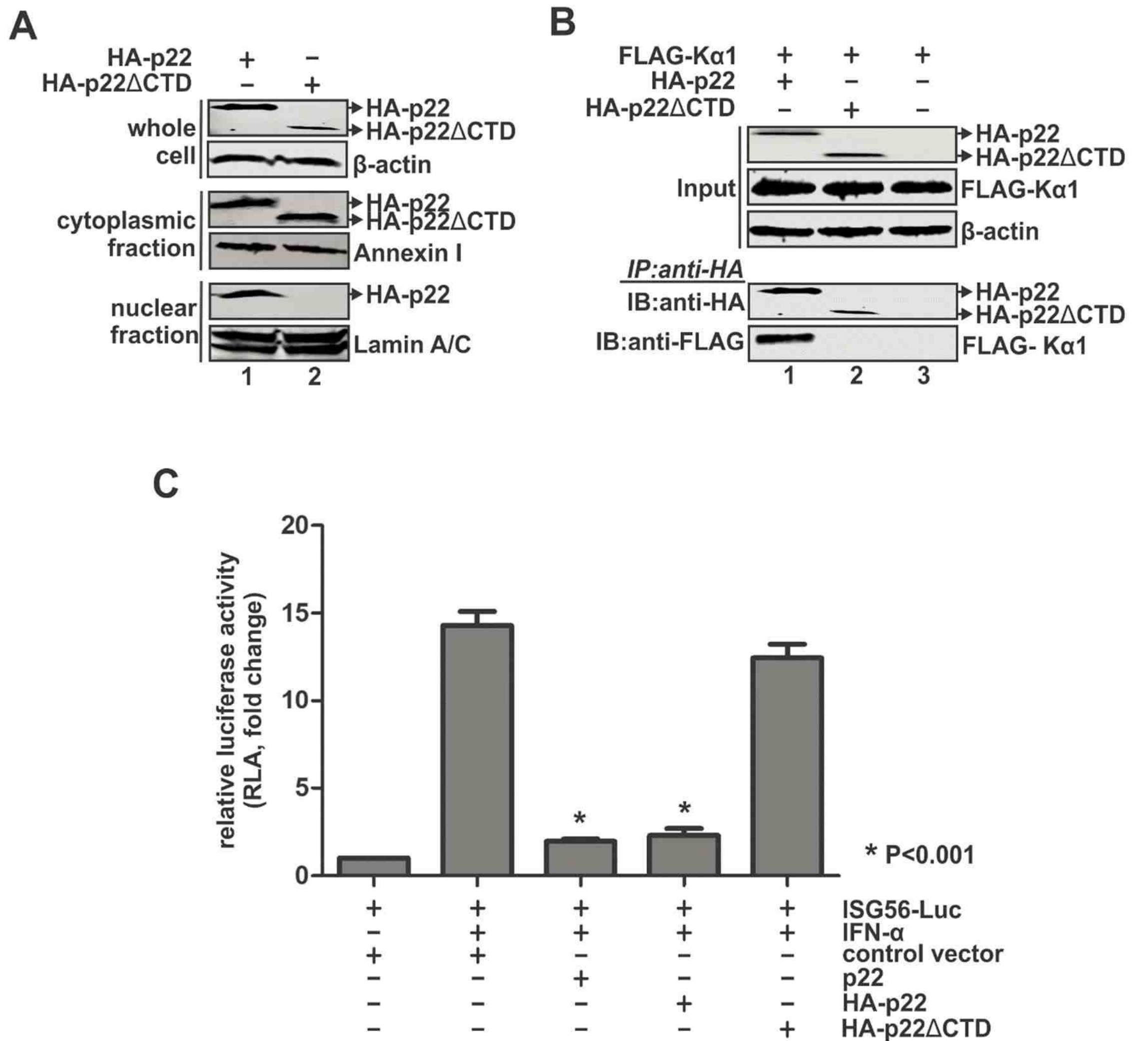


Figure 14: The CTD domain is required for the p22-mediated inhibition of IFN signaling (A) HepG2 cells in a 6-wellplate were transfected with plasmid expressing HA-p22 or HA-p22ΔCTD for 5 days and subjected to cell fractionation assay. The presence of full-length and CTD-truncated HA-p22 in whole cell lysate and the subcellular fractions were assessed by Western blot using anti-HA antibody. β-actin served as loading control for whole cell lysate. Annexin I and Lamin A/C served as marker and loading control for cytoplasmic and nuclear lysate, respectively. (B) 293T cells were in 6-well-plate were co-transfected with FLAG-Kα1 expressing plasmid plus HA-p22 or HA-p22ΔCTD expressing plasmids, or

control vector, as indicated, for 5 days. The transfected cells were then lysed and subjected to co-immunoprecipitation with anti-HA antibody-coated beads, and the co-immunoprecipitated FLAG-Kα1 was detected by Western blot using anti-FLAG antibody. (C) HepG2 cells in 96-well-plate were co-transfected with ISG56-Luc and control vector, or p22, or HA-p22, or HA-p22ΔCTD as indicated, plus pRL-CMV. 5 days later, the transfected cells were left untreated or treated with IFN-α (1,000 IU/ml) for 18 h. The cells were then harvested for dual luciferase assay. *Renilla* luciferase activity served as the internal control to normalize transfection efficiency. The relative Firefly luciferase activities in each sample were plotted as fold changes versus the control group without IFN-α treatment (mean ± SD, n=3).

3.6 FUNCTIONS OF NUCLEAR p22

3.6.1 p22 BINDS TO cccDNA

In order to investigate the ability to p22 to bind to HBV cccDNA we used the the HepBHAe82 cells released for 14 days prior to fixing and collection of the chromatin. Unreleased HepBHAe82 cells were used as negative control. Chromatin Immunoprecipitation (ChIP) was carried out using antibodies against anti-HA, anti-core, anti-H3 and anti-H3K27Ac and anti-IgG to pull down DNA associated with HA-p22, core, and histone 3 (H3) and DNA expressing the H3K27Ac post-translational modification respectively. The IgG pulldown served as a negative control for our technique. The pulled -down proteinase K -treated DNA was amplified using regular and quantitative PCR with primers specific for HBV cccDNA. Figure 15A shows the primers designed specifically to amplify the HBV cccDNA. Figure 15B shows a post-ChIP pulled-down cccDNA from 14-day released HepBHAe82 cells amplified using a regular PCR followed by agarose gel electrophoresis confirming the ability of both p22 and core binding to HBV cccDNA with Histone 3 serving as a positive control and IgG serving as a negative control. Figure 15C confirms the results in Figure 15B where the pulled-down cccDNA was amplified using qPCR data confirming a similar binding to p22 with H3K27Ac serving as the positive control. Unreleased HepBHAe82 serve as a negative control showing absence of HBV DNA and DNA binding to HA-p22.

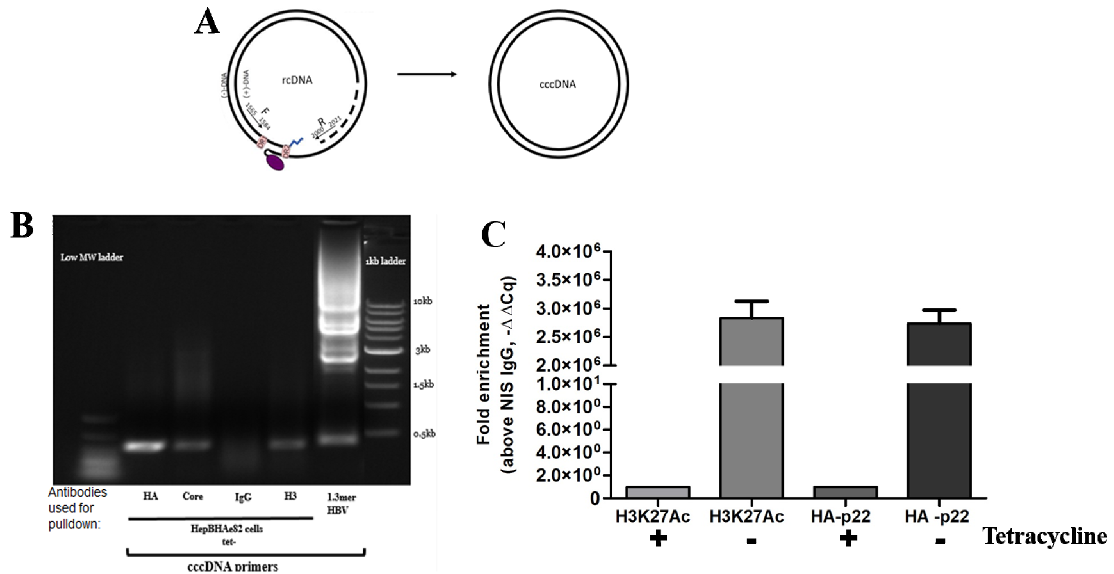


Figure 15: p22 binds to cccDNA

(A) Shows the location of the primer sequences designed to specifically amplify newly-formed cccDNA only (and not the integrated copy). (B) and (C) HepBHAe82 cells were released for 14 days to allow for HBV replication. Chromatin immunoprecipitation was carried out with the released cells with anti-HA, anti-core, anti-IgG, anti-H3 and anti-H3K27Ac to pull down bound cccDNA (if any) with HA-p22, core, IgG, Histone3 and H3K27Ac PTM. IgG served as negative control and Histone3 and H3K27Ac PTM served as positive control for our experiment. Pulled –down cccDNA was specifically amplified and was analysed either through a PCR reaction and a polyacrylamide gel electrophoresis (B) or through qPCR analysis (C).

3.6.2 LOSS OF p22 NEGATIVELY AFFECTS cccDNA STABILITY AND/OR TRANSCRIPTION

To further investigate the function behind binding of p22 onto HBV cccDNA, we opted to study the cccDNA copy number to understand effect of p22on cccDNA stability and the precore mRNA and total HBV RNA levels to understand cccDNA transcription in presence or absence of p22. For this study, we resorted to the infection model using HepG2-NTCP12 cells and PXB cells. We utilized two types of p22-null viruses, one contains a start codon mutation of the precore ORF in viral genome, and the second one harbors the precore G1896A stop codon mutation. The wildtype and p22-null virus were used to infect the HepG2-NTCP12 and PXB cells with same inoculum size for 7 days, 3TC was included during the infection to prevent de novo HBV DNA replication and the intracellular cccDNA amplification. With the Δe or G1896A virus infection, we saw a trend towards decreased cccDNA copy number (Figure 16 A, D and G) as well as with total HBV RNA (Fig 16 B, E and H) and precore RNA (Figure 16 C, F and I) levels, however the seen decrease wasn't statistically significant. This slight decrease may be attributed to the presence of core protein in the system even in absence of p22 since both core protein and p22 share similar sequence. Further studies involving core and precore-null (ΔcΔe) virus infection and analysis of cccDNA stability and transcription are currently underway. We are also working towards identifying p22's binding sequence on cccDNA to better tease out p22's role on cccDNA formation, maintenance and/or transcription.

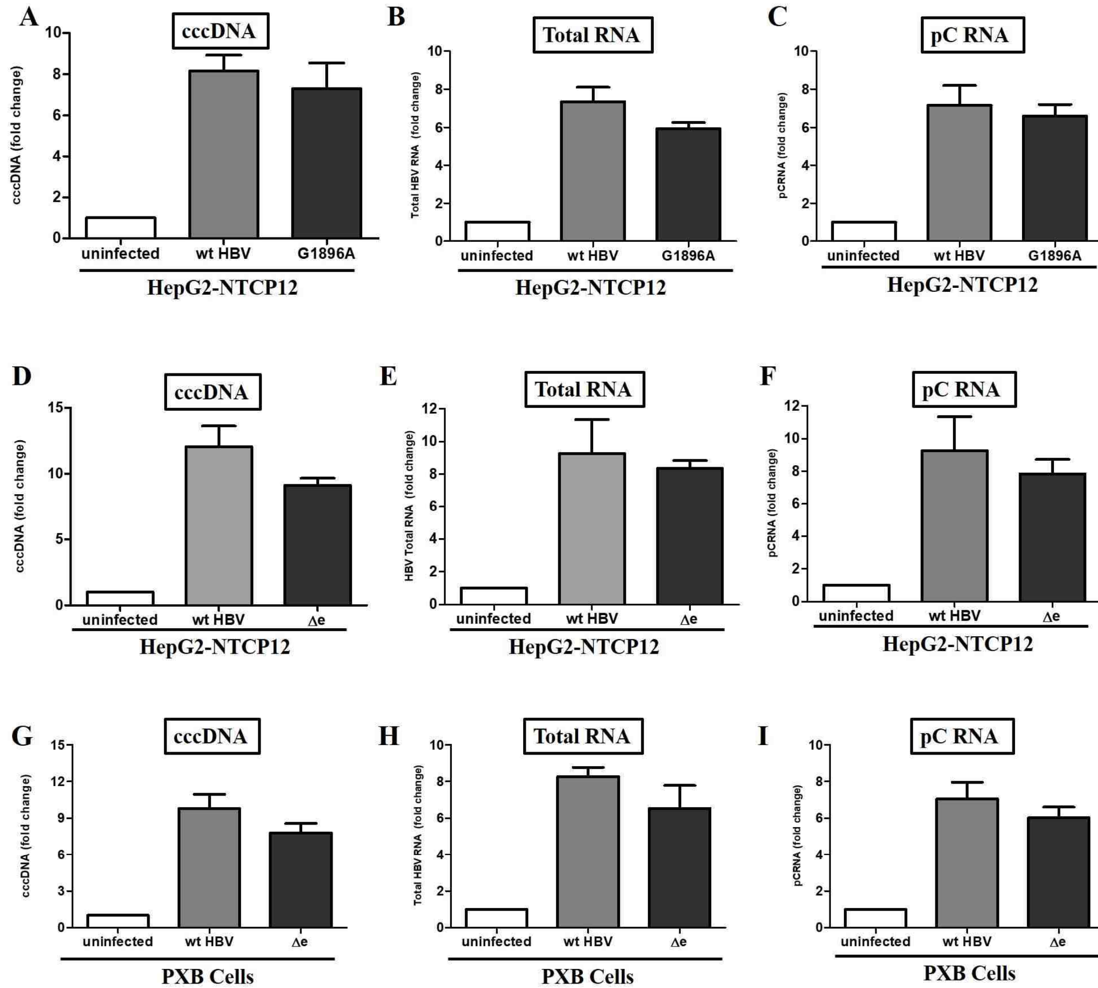


Figure 16: p22 negatively affects cccDNA formation and/or transcription

HepG2-NTCP12 cells in 96-well-plate or PXB cells in 24-well plate were left uninfected or were infected with the wildtype HBV (for both HepG2-NTCP12 and PXB cell infections), G1896A mutant (for HepG2-NTCP12 cell infection only, A-C) or A1814T precore-null viruses (for both HepG2-NTCP12, D-F and PXB cell infections, G-I), respectively, at 500 VGE/cell for 24 hours. The infected cells were then cultured in the presence of 10 μM of 3TC for 7 days. cccDNA levels (A,D and G), Total HBV RNA levels (B, E and H) and pCRNA levels (C, F and I) were assessed via qPCR (mean±SD, n=3).

3.6.3 NUCLEAR p22 AND CORE CAN BIND TO THE ISRE ELEMENTS OF ISGS

Previously in Figure 10 we have seen that core is able to show a similar decrease in ISG expression like p22. However, when tested for nuclear translocation inhibition like p22, we found that core doesn't cause the decrease in ISG expression in the similar mechanism. Further, p22 is unable to inhibit ISG expression completely and nuclear translocation inhibition of the pSTATs isn't completely blocked by p22, in spite of a strong decrease in the ISRE activity in presence of p22. Thus, we hypothesized that nuclear core and nuclear p22 are able to bind to ISRE of the IFN-stimulated ISGs and negatively affect ISG expression.

Using 14-16 days released HepBHAe82 cells and treated with IFN-α for 10 hours (Figure 17 A-D) or 18 hours (Figure 17 E-H) were subjected to ChIP using antibodies against HA-tag, core, and pSTAT1, H3K27Ac and IgG where pSTAT1 and H3K27Ac serve as the positive control and IgG as the negative control. As seen in Figure 17, both the p22 (A and E) and the core (B and F) were found to be able to bind to MxA, ISG56, IRF9 and ISRE-ISG56. High binding of pSTAT1(C and G) and increased presence of H3K27Ac (D and F) modification on the same regions of these ISGs and ISRE-ISG56 served as a positive control. However, HBV core and p22 proteins didn't show a significant binding to OAS2 in spite of high pSTAT1 binding and presence of H3K27Ac modification. This also explains the reduced inhibition in OAS2 expression seen in the infection model with wildtype virus infection upon IFN-α induction. Similar results were seen when the IFN-α induction was carried out for 10 hours or 18 hours.

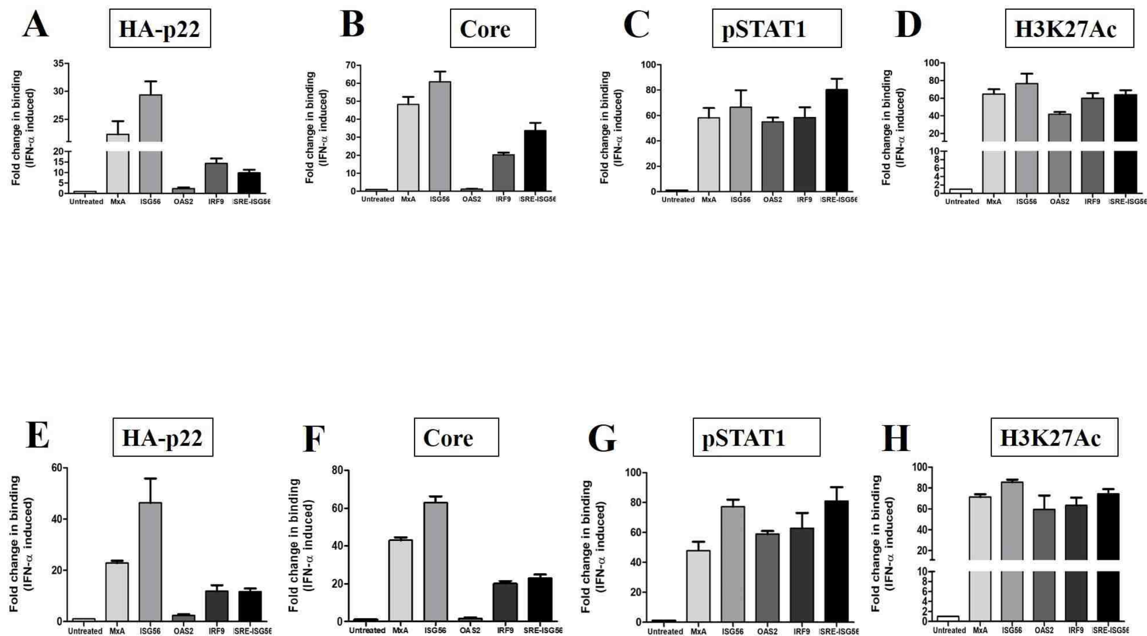


Figure 17: Nuclear p22 and core bind to promoter regions of ISGs

HepBHAe82 cells were released for 14 days to allow for HBV replication before 10 hours (A-D) or 18 hours (E-H) treatment with IFN-α. Antibodies against HA, core, pSTAT1 and H3K27Ac PTM were used to carry out chromatin immunoprecipitation to pull down ISG-promoter/ISG sequences bound by HA-p22 and core with pSTAT1 and H3K27Ac PTM serving as positive control. Binding to sequences specific to 5' sequences of 4 ISGs- MxA, ISG56, OAS2 and IRF9 and for ISRE of ISG56 family were tested (mean ± SD, n=3).

CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

HBeAg is a serological marker of hepatitis B and a known non-structural protein of HBV. The circulating HBeAg has been reported to regulate various host immune responses and is often associated with high viremia in chronic hepatitis B patients, suggesting a pro-viral activity of HBeAg (31, 172-174). However, the function of intracellular precore protein p22, the precursor for HBeAg, remains largely elusive. In the present study, we made an attempt at identifying if p22's formation uses ERAD to move into the cytosol, characterizing p22's ability to support replication and to identify the function for its cytosolic form. With overexpression of dominant negative form of p97 translocon on the ER membrane, we showed that p22 is still able to transport to the cytosol suggesting use of an ERAD-independent mechanism for retro-translocation. Moreover, the p22ΔCTD mutant was also able to retro-translocate to the cytosol ruling out the possibility of use of CPP-mediated translocation. However, another hypothesis to explain the seen results could be that biogenesis of p22 probably occurs on the ER outer membrane or in the cytosol and a larger portion of the newly formed p22 goes into the ER lumen through Sec61 to follow the secretory pathway and get released as HBeAg while the rest of it remains in the cytosol or translocates to the nucleus. The other possibility is that on the signal peptide cleavage and co-translation occurs through the Sec61 translocon on the ER membrane, a small portion of newly formed p22 uses the same translocon to get released back into the cytosol. Studies with Sec61 mutant and biogenesis kinetics of p22 are currently underway in our laboratory. Further, our results reconfirmed the presence of intracellular p22 as well as its cytosolic and nuclear distribution (78, 80, 175) (Figures 5

and 6). The localization study for p22 was done using stable cell line model instead of infection model because of known caveats with the later model. The stable cell line models-HBe4 provided a constitutive expression of HA-p22 from the transgene while HepBHAe82 provided a cccDNA-dependent expression of HA-p22- this allows for a distinction of p22 from core because of identification through HA—tag instead of antibodies directed towards the CTD of p22 which is shared by core protein as well. However, for the infection model, we lack a HA-p22 expressing virion hence any identification of p22 will likely be representative of a mixture of p22 and core because the antibody for p22 will cross-react. Efforts have been made previously to design an antibody against the unique extended NTD of p22 but with to no success. Thus, the presence and the identification of HA-tag in our system is extremely important in distinguishing p22 from core protein within the system.

Although the cytosolic p22 shares the exact same amino acid sequence as the core protein except for an 10 aa N-terminal extension, it fails to form a capsid-like structure and support HBV DNA replication (Fig. 8A), which is consistent with previous studies showing precore is not required for HBV infection in vivo or replication in vitro(176, 177), though it can form heterocapsid with core protein(178, 179). It has been shown that the presence of Cys -7 in the N-terminal precore domain of HBeAg causes prefers the formation of intra-disulfide bond between the -7 and 61 cysteine over inter-disulfide bond as seen in the case of the core protein, resulting in HBeAg dimerization in an inverted orientation relative to core dimer, and thus precluding capsid formation from HBeAg. In line with this, the bacterially expressed HBeAg/p17 with -7 cysteine-to-glutamine (C-7Q) or under reducing conditions restores particle formation(81, 101). However, we found that p22 with C-7Q mutation remains non-particulate in HepG2 cells and does not support HBV

DNA replication (Figure 8B), indicating that the N-terminally extended precore domain blocks p22 to assemble into capsid-like structures in cell cultures. However, whether the intracellular p22 exists as monomeric or dimeric form remains unknown.

A previous study with hepatitis B patient serum samples suggested that a precore-derived aberrant 22kD protein devoid of the CTD domain but harboring the N-terminal signal peptide, termed p22cr, could self-assemble into HBV genome-free capsid and acquire envelope to be secreted as empty virion(180). In our study, we did not detect capsid formation in cells from precore expressed alone or in the context of core-null HBV transfection (Figure 8). It is unclear whether the p22cr species is present in our experimental system or not, but nonetheless, we were always able to detect the canonical CTD-containing p22.

Despite the dispensable role of precore in HBV replication cycle, the observed high viremia and low immune system activation in HBeAg(+) chronic hepatitis B patients directs to the role of HBeAg and/or p22 in relation to the regulation of host defense and virus persistence (31, 44, 172, 174). Furthermore, it has been reported that HBeAg(-) patients responds better to standard IFN-α therapy over HBeAg(+) patients(117, 118, 181-183), suggesting that either the HBeAg or its precursor, p22, might have an inhibitory effect that prevents the IFN-therapy to work to its full potential in the HBeAg-positive patients. Following this direction, we, for the first time, performed a comparative analysis of ISG transcriptome profile in consecutive liver biopsies from HBeAg(+) and HBeAg(-) patients at baseline and the endpoint of 24-week IFN-α therapy by RNA-seq, and found a statistically stronger induction of ISGs in HBeAg(-) patients compared to HBeAg(+) patients (Figure 9), indicating a potential inhibitory effect of HBeAg and/or p22 on IFN-

signaling. Furthermore, cell-based ISG56 promoter reporter assay revealed that the intracellular p22, but not the exogenously treated HBeAg, is able to suppress IFN-α activity (Figure 10A). The mRNA levels of MxA and ISG56 were found to be reduced in presence of p22 (Figure 10B), further confirming that p22 inhibits IFN-α-induced JAK-STAT signaling and ISG expression. In addition, infection of HepG2-NTCP12 cells with the precore-null virus followed by IFN-α treatment led to similar levels of ISG expression (MxA, ISG56, OAS2, IRF9) compared to uninfected control, while the ISG induction levels were significantly reduced in the wild-type HBV infected cells, which validated role of p22 in antagonizing IFN signaling in a more physiologically relevant setting (Figure 11).

In order to elucidate the mechanism of p22-mediated inhibition of IFN signaling, we examined the effect of p22 on cellular STAT1/2 phosphorylation and nuclear translocation upon IFN-α treatment. The results demonstrated that p22 does not affect the IFN-α-activated tyrosine phosphorylation of STAT1/2, but significantly downregulates the nuclear importation of pSTAT1/2 (Figure 21). We first performed the co-immunoprecipitation assay of p22 and pSTAT1/2 but did not detect obvious interaction between p22 and pSTAT1/2. Considering that pSTAT1 engages a subset of nuclear localization signal (NLS) receptors, including Kα1, for nuclear entry to bind to the ISRE for activation of ISG expression, we next examined the interaction between p22 and Kα1. The results demonstrate that p22 efficiently binds to Kα1 and thus competes with pSTAT1 to interact with the same receptor and prevent the nuclear translocation of pSTAT1 (Figure 13). As expected, the NLS-containing CTD domain of p22 is responsible for Kα1 binding and the deletion of CTD abrogates the nuclear transportation of p22 and the inhibitory

effect of p22 on IFN-α signaling (Figure 14). Thus, we conclude that HBV p22 inhibits IFN-α-elicited JAK-STAT pathway by blocking pSTAT nuclear translocation through competing Kα1 binding (Figure 18). Prevention of nuclear import of activated STAT1 is employed by many other viruses to antagonize IFN-mediated antiviral innate defense (63). For instance, Ebola virus protein VP24 has been reported to inhibit STAT1 nuclear translocation by interacting with karyopherin α1, α5, and α6 (64-66). However, except for Kα1, we did not detect the binding of p22 with other karyopherin α proteins using co-immunoprecipitation (data not shown). The preferred interaction of p22 with Kα1 over other karyopherin α proteins may be due to that the NLS of p22 possesses optimal affinity to Kα1's binding sequence or structure and may partly explain the observed incomplete inhibition of pSTAT1 nuclear translocation (Figure 12 C). On the other hand, it may also ensure a selective inhibition of STAT nuclear translocation without altering the transport of other nuclear proteins essential for virus replication and cell viability.

It is worth noting that HBV core protein also exhibits inhibitory activity against IFN-α-induced activation of ISG56 promoter and ISG production (Figure 10). However, core is unable to inhibit the nuclear translocation of pSTAT1 and pSTAT2 (Figure 12 D), suggesting that core and p22 inhibit IFN-α signaling via distinct mechanisms. Although core shares the entire amino acid sequence with p22 including the CTD domain, core predominantly exists in a capsid structure where the CTD domains are localized on the interior side of capsid shell and are inaccessible to karyopherins (53, 67). We, thus, reason that core protein may exert its inhibition of ISG expression in the nucleus. It has been shown that HBV core can shuttle between cytoplasm and nucleus via an NLS and nuclear export signal (NES) (16, 68, 69), and the nuclear core plays a role in regulating host gene

expression and viral episomal integrity (70, 71). Interestingly, a substantial portion of p22 is also found in the nucleus (15) (Figure 6). Hence, we investigated the potential inhibitory effect of nuclear p22 and/or core on ISG expression at the late stage of JAK-STAT cascade, such as competitive binding to the conserved ISRE motif or specific promoter of ISGs, leading to decreased transcription of the latter, or affecting the epigenetic profile of the ISG promoters negatively. Our ChIP experiment results showed increased binding of the nuclear core and the nuclear p22 to the ISRE site of ISG56 family and 5' end of ISGs, such as, MxA, ISG56, IRF9 etc (Figure 17). High abundance of pSTAT1 protein and H3K27Ac modification on the same regions in IFN-induced cells served as a positive control. The binding probably leads to the inhibition of transcription of the ISGs explaining the reduced relative expression of the ISGs seen in the overexpression system, especially for the core protein, in Figure 10B. Hence, we hypothesize the possibility of change in the epigenetic profile of these ISG promoters on IFN-induction in presence of HBV core and/or p22 proteins which may have a negative effect on the transcription of these ISGs. This study is currently underway where we are testing the change in the epigenetic profile of the ISG promoters in absence of only core, only p22 and both core and p22 with respect to the wildtype.

The cccDNA plays a pivotal role in the HBV life cycle and is established in the nucleus upon initial infection. Once established, cccDNA functions as the persistent form of the HBV genome. Moreover, histone modifications of the cccDNA minichromosome have been shown to regulate cccDNA transcription(184). Our previous results have shown the presence of nuclear p22. The CTD of the core protein is arginine-rich (185) and there has been speculation regarding core protein associating with HBV genome replicative

intermediates including cccDNA minichromosomes (63) and may play a role in the regulation of cccDNA transcription through chromatin rearrangement and/or epigenetic mechanisms. Host cellular transcription factors, co-activators, co-repressors, chromatin-modifying enzymes, as well as viral proteins, such as core and X proteins, have also been demonstrated to be recruited to cccDNA minichromosome under certain conditions to regulate cccDNA transcription activity(186). Considering that p22 shares the same CTD with core (Figure 3), we hypothesized that p22 may be able to bind to cccDNA as well and if so, what would the implications of that binding with respect to stability and transcription of cccDNA. We were able to show that, as per hypothesis, both the nuclear core and p22 proteins are also able to bind to cccDNA (Figure 15 B and C). Moreover, A1814T and G1896A mutant virus infection resulted in a minimal decrease in cccDNA amount and its downstream transcription products, precore RNA and total HBV RNA compared to the wt virus infection (Figure 16) suggesting an important role of p22 in cccDNA biology. The seen decrease, however, wasn't significant and currently, we are studying with a core and p22-double null- mutant virus to test if the two similar proteins have a synergistic effect on cccDNA formation, stability and /or transcription. The nuclear core and precore can bind to promoters of ISGS as well as to HBV cccDNA (Figure 18).

Taken together, our study gives a further inkling into the biological functions of the enigmatic intracellular HBV precore protein in viral pathogenesis. Though precore or HBeAg is not absolutely required for HBV infection, a growing body of evidence suggest that it serves as an immunomodulator to maintain persistent HBV infection, and the intracellular precore (p22) mainly antagonizes the innate immune responses (7, 20, 26). Further, precore can give us an insight into cccDNA biology that remains poorly

understood. Therefore, precore and HBeAg may hold promise for being considered as an additional antiviral target in developing novel hepatitis B therapeutics or at least, to improve the effectiveness of IFN-based immunomodulatory therapy especially due to ability of p22 and core to bind to promoter of ISGs and causing an additional blockade to interferon response besides p22 inhibiting IFN signaling. Further assessment of the effect of nuclear p22 and nuclear core (p21) protein on ISRE activity and potential epigenetic regulations will help to better understand the modes employed by p22 and core to counteract/inhibit host interferon response in infected cells.

Our studies have shown the ability pf p22 to bind to cccDNA which in future can lead to some interesting studies on the effect of these viral proteins on maintaining cccDNA stability and form instead of just acting as a scaffold proteins. The most imminent step is to map the p22-binding sequence on cccDNA using ChIP-seq which can provide us information in generating a hypothesis on binding of p22 onto cccDNA depending the region of cccDNA the former binds the latter. Further, the negatively affected cccDNA transcription in absence of p22 can direct towards an effect on the cccDNA epigenetics. We need to elucidate the role of nuclear p22 and/or nuclear core on HBV cccDNA epigenetics. Core-minus only and core & precore - double minus virus infections to elucidate the synergistic effect (if any) of nuclear core and nuclear p22 on cccDNA stability and/or transcription will further shed light on the importance of shared sequence of the two viral proteins and highlight the similarity between them. Core and p22 CTD are known to have few major phosphorylation sites and a study of the potential role of CTD phosphorylation in p22 subcellular localization and functions will further help us identify the similarities between p22/precore and core.

The biogenesis of p22 is yet another under-studied area which might add to the current knowledge of molecular biology in biogenesis of proteins within the cell. Our studies show that p22 doesn't use the ERAD to retro-translocate to cytosol. We need to identify the rate-determining step for the sorting of p22 along the ER-Golgi with the use of Brefeldin A (BFA) to blocking ER to Golgi transport and elucidate the role of Sec61 and Signal Peptidase (SPase) in cytoplasmic retro-translocation route for p22 through allosteric inhibitors and siRNA knockdown and its effect on p22 microsomal and cytosolic distribution to determine if p22 in fact enters the ER lumen.

Study of precore biology adds a step forward to the knowledge of HBV. HBV expresses only seven proteins and where many of them being multi-functional. Precore protein is one of the most under-studied proteins that might be target for development of small molecule inhibitors for an effect on cccDNA, the ultimate formidable player in the HBV infection, an antiviral target in developing novel hepatitis B therapeutics or at least and more importantly improve the effectiveness of IFN-based immunomodulatory therapy.

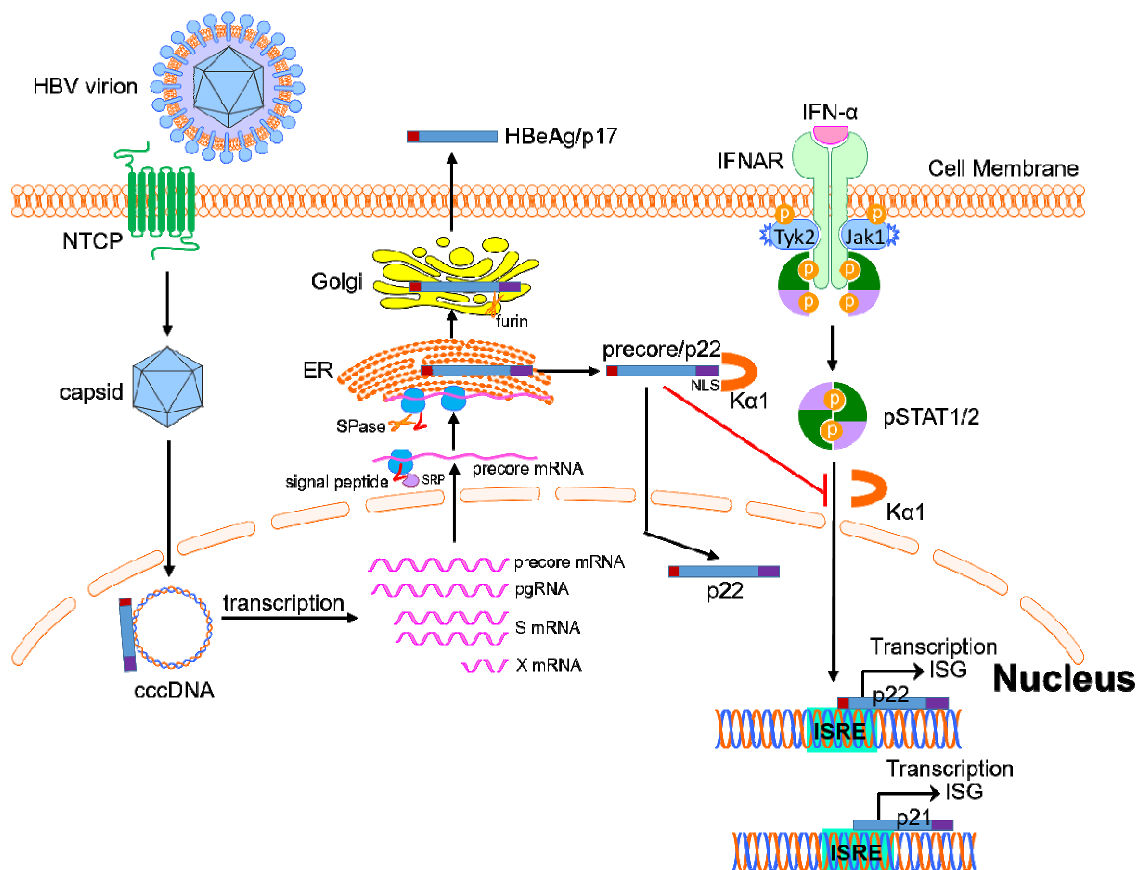


Figure 18: Model of action of p22-mediated inhibition of IFN-α Signaling

HBV virion infects hepatocyte *via* NTCP-mediated entry, followed by nuclear import of nucleocapsid and the establishment of cccDNA episome. cccDNA serves as transcription template to produce viral mRNAs. During the translation of precore protein from the 3.5kb precore mRNA, the initially translated N-terminal signal peptide is recognized by cellular signal recognition particle (SRP) receptor and directed to rough ER. After the cleavage of signal peptide by ER-associated signal peptidase (SPase), the translation resumes and p22 is synthesized and enters ER lumen. Subsequently, p22 is sorted into Golgi apparatus, where the CTD is cleaved off by Golgi resident endopeptidase furin to generate the secreted

HBeAg/p17. In the meantime, a portion of p22 is retro-translocated into cytosol from ER, and can be further imported into nucleus due to the presence of NLS motif on CTD domain, which is recognized by cellular nuclear transportation receptor karyopherin α1 (Kα1). When the cell receives IFN-α treatment, the cytoplasmic p22 can blunt the IFN-α-elicited JAK-STAT pathway through competing the binding of Kα1 with activated pSTAT, leading to a downregulated IFN signaling and ISG expression. This mode of action represents a viral strategy to evade the innate antiviral response.

The nuclear core and the nuclear P22 upon entry is able to bind to the ISG's at the 5' end near or onto the ISRE promoters regions. The binding probably leads to the inhibition of transcription of the ISGs explaining the reduced relative expressions of the ISGs seen in the overexpression system in Figure 9B. The nuclear p22 is also able to bind to cccDNA and its absence has a negative effect on cccDNA formation/stability and/or transcription suggesting an important role of p22 in cccDNA biology.

Table A1: Primers for cellular and viral (precore and cccDNA) gene qPCR

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	qPCR probe sequence (5'→3')
MxA	TGATCCAGCTGCTGCATCCC	GGCGCACCTTCTCCTCATAC	
ISG56	TCTCAGAGGAGCCTGGCTAAG	CCACACTGTATTTGGTGTCTAGG	
OAS2	AACTGCTTCCGACAATCAAC	CCTCCTTCTCCCTCCAAAA	
IRF9	TTCAGGATGGCCTCAGGCAAAGTA	GAACAAGTCTATTTCCATGGAGACG	
ISG20	TAGCCGCTCATGTCCTCTTT	TGAGGGAGAGATCACCGATT	
cccDNA	TCATCTGCCGGACCGTGTGC	TCCCGATACAGAGCTGAGGCGG	TTCAAGCCTCCAAGCTGTGCCTTGGGTGGC-TAMRA
pCRNA	G TAG GCA TAA ATT GGT CTG	GTG CAG TAT GGT GAG GTG AAC AAT	CTC AGG AGA CTC TAA GGC TTC CCG ATA CAG
Total Hirt HBV	CCGTCTGTGCCTTCTCATCTG	AGTCCAAGAGTYCTCTTATGYAAGACCTT	CCGTGTGCACTTCGCTTCACCTCTGC -TAMRA
COX3,Mito DNA	CCCTCTCGGCCCTCCTAATAACCTG	GCCTTCTCGTATAACATCGCGTCA	
β-actin	CCTGGCACCCAGCACAATGA	ACTAAGTCATAGTCCGCCTAGA	

Table A2: Plasmids

#	Plasmid Name	Description	References
1.	pHBV1.3	Expression plasmid under HBV promoters containing 1.3 copies of the HBV genome	150, 151-154
2.	CMVHBV	Expression plasmid under CMV-IE promoters containing a copy of the HBV genome	150, 151-154
3.	pHBV1.3ΔC	Expression plasmid under HBV promoters containing 1.3 copies of the HBV genome with mutation of the start codon (ATG to ATA) of the core protein open reading frame (ORF)	155
4.	pCMVHBVΔe-A1814T	The sequence between the RsrII and AflII restriction sites on pCMVHBV with was replaced with a synthetic sequence containing the precore start codon mutation (AUG to TTG [A1814T]	192
5.	pTREHBV-HAe	An in-frame HA epitope sequence was introduced into the coding sequence of precore domain without disrupting the predicted ribonucleotide base pairing in the overlapped coding sequence for epsilon stem-loop region, a DNA fragment which contains HA coding sequence (GTGGACATCTACCCATACGACGTTCCAGATTACGC TGGC, HA sequence is underlined) was inserted into pTREHBVDES at a position immediately upstream of the start codon of core ORF (between HBV nt 1902 and nt 1903, genotype D, subtype ayw) on TREHBVDES	156
6.	pTREHBVΔHAe	the sequence between the SacI and BspEI restriction sites on pTREHBV-HAe was replaced with a synthetic sequence containing the precore stop codon mutation G1896A	192
7.	pcHBc	pcDNA3.1-derived HBV core expression vector with HBV core ORF containing sequence fragment (nt 1903–	156

		2606/1573–1926) being placed under the control of CMV-IE promoter	
8.	pcHBe	pcDNA3.1-derived HBV HBeAg expression vector with HBV precore ORF containing sequence (nt 1816–2606/1573–1926) under the control of CMV-IE promoter	156
9.	pcHA-HBe	pcHBe with an in-frame HA epitope sequence into the coding sequence of precore domain at a position immediately upstream of the start codon of core ORF	156
10.	pcHBeΔSP	N-terminal signal peptide (SP; aa 2 to 19)-coding sequence deletion on pcHBe	192
11.	pcHBe-C-7Q	Expression plasmid for Precore gene of HBV with -7Cys mutated to Gln	192
12.	pcHA-HBeΔCTD	Deletion of the HBV precore/core C-terminal domain (CTD; aa 149 to 183)-coding sequence on pcHA-HBE	192
13.	pLMS	Expressing the HBV large (L), middle (M), and small (S) envelope proteins	157
14.	pCMV-FLAG-Pol	Expressing 3×FLAG-tagged HBV Pol under CMV-IE promoter	158
15.	pFLI-154 HBx	Expressing FLAG-tagged HBx	159
16.	pISG56-Luc	Firefly luciferase reporter plasmid under IS56 (ISRE-bearing) promoter	160
17.	pFLAG-Kα1-6	N-terminal FLAG-tagged karyopherin-α1 to -6	161-162
18.	pHA-VP24	HA-tagged Ebola virus VP24	161-162

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192. Mitra B, Wang J, Kim ES, Mao R, Dong M, Liu Y, Zhnag J, Guo H. 2019. Hepatitis B Virus precore protein p22 inhibits alpha interferon signaling by blocking STAT nuclear translocation. J Virol 93(13): e00196-19

CURRICULUM VITAE

Bidisha Mitra

Education

PhD | 2014 - 2019 | Indiana University, Indianapolis

· Major: Microbiology and Immunology

· Minor: Cancer Biology

Bachelor of Science | 2010 - 2014 | St. Xavier's College (Autonomous), Kolkata, India

· Major: Microbiology

· Minor: Chemistry and Physics

Publications

- **B. Mitra**, J. Wang, E.S. Kim, R. Mao, M. Dong, Y. Liu, J. Zhang, H. Guo (2019). Hepatitis B Virus precore protein p22 inhibits interferon-alpha signaling by blocking STAT nuclear translocation. J. Virol. 93(13). pii:e00196-19 PMID: 30998767
- F. Yang, X. Yu, M. Zhu, C. Zhou, H. Zhu, R. Mao, Y. Qin, J. Yu, Y. Zhang, Z. Shen, F. Li, P. Zhou, J. Wang, S. Yang, J. Li, X. Qi, M. Dong, **B. Mitra**, G. Zhao, Y. Huang, H. Guo, B. Wang, J. Zhang (2019). Hepatitis B e antigen induces the expansion of monocytic myeloid-derived suppressor cells to dampen T-cell function in chronic hepatitis B virus infection. PLoS Pathogen 15(4):e1007690 PMID: 30998767
- **B. Mitra**, R. Thapa, H. Guo, T. Block (2018). Host functions used by hepatitis B virus to complete its life cycle: Implications for developing host-targeting agents to treat chronic hepatitis B. Antiviral Res. 158:185-198 PMID: 30145242

- Q. Long, R. Yan, J Hu, D. Cai, **B. Mitra**, E. Kim, A. Marchetti, H. Zhang, S. Wang, Y. Liu, A. Huang, H. Guo (2017) The Role of Host DNA ligases in Hepadnavirus Covalently Closed Circular DNA Formation. PLOS Pathogens 13(12): e1006784 PMID:29287110
- Y Liu, H. Nie, R. Mao, **B. Mitra**, D. Cai, R. Yan, J.T. Guo, T.M. Block, N. Mechti, H. Guo (**2017**) Interferon-inducible ribonuclease ISG20 inhibits Hepatitis B virus replication through directly binding to the epsilon stem-loop structure of viral RNA. PLOS Pathogens 13(4): e1006296 PMID: 28399146
- **B. Mitra**, H. Guo (**2016**) Hepatitis B virus X protein crosses out Smc5/6 complex to maintain covalently closed circular DNA transcription. Hepatology 64(6): 2246-2249 PMID: 27639252
- **B. Mitra**, A. Chatterjee, T. Mookherjee, M. Basu, S. Das, A. K. Mitra (**2014**) Study of Host (*Michelia champaca*) and Pathogen (*Phomopsis micheliae*) interaction. IJAPBC 3(4): 884-900
- Saha, A.K. Mitra, S.S. Chowdhury, **B. Mitra** (**2013**) Chromium Uptake by a Bacteria Isolated From *Lemna* Rhizosphere in a Lentic Ecosystem. IMEJ 2(1):67-73
- R. Das, I. Saha, **B. Mitra**, D. Bhowmik, A. Chakraborty, D. Sengupta, S. Roy, S. Chowdhury, A. K. Mitra, R. Chakraborty, S. Mukherjee (**2012**) Bioaccumulation of Chromium by Aquatic Hydrophyte *Lemna* sp. and its Associated Rhizosphere Bacteria. Aviskaar Vol 4

Abstracts

- Mitra, B., Mao R, Kim E, Wang J, Liu Y, Zhang J, Guo H. Biogenesis and Function of Intracellular HBeAg. The 2018 International HBV Meeting. The Molecular Biology of Hepatitis B Viruses. October 3–6, Taormina, Palazzo dei Congressi, Italy.
- E. Kim, J. Zhou, A. Marchetti, B. Mitra, D. Cai, Y. Liu, M. Wang, H. Guo. Epigenetic regulation of HBV cccDNA transcription by interplay of HBx and HMGB1. The 2018 International HBV Meeting. The Molecular Biology of Hepatitis B Viruses. October 3–6, Taormina, Palazzo dei Congressi, Italy.
- E. Kim, J. Zhou, A. Marchetti, B. Mitra, D. Cai, Y. Liu, M. Wang, H. Guo. Chromatin Control of Viral Infection Workshop. Epigenetic regulation of HBV cccDNA transcription through interplay of HBx and HMGB1 proteins. September 6–7, William H. Natcher
- Mitra, B., Mao R, Kim E, Wang J, Liu Y, Zhang J, Guo H. Biogenesis and Function of Intracellular HBeAg. 2018 International Liver Congress, Paris, France.
- Mitra, B., Mao R, Kim E, Wang J, Liu Y, Zhang J, Guo H. Biogenesis and Function of Intracellular HBeAg. 2017 International HBV Meeting, Washington DC
- Kim E, Zhou J, Cai D, Mitra B, Liu Y,Guo H, Investigating the Role of X protein in HBV cccDNA transcription. 2017 International HBV Meeting, Washington DC
- Mitra, B., Liu Y, Cai D, Yan R, Mao R, Guo H. The biological functions of intracellular BHeAg. 2016 International HBV Meeting, Seoul, South Korea

Conferences and Presentations

- 2018 International HBV meeting. The Molecular Biology of Hepatitis B Viruses. Taormina, Italy

  Oral Presentation: Biogenesis and Functions of Intracellular Hepatitis B e Antigen
- 2018 International liver Congress, EASL, Paris, France

  Poster Presentation: Biogenesis and Functions of Intracellular Hepatitis B e Antigen
- 2017 Fourth Midwestern Membrane Trafficking and Signaling Symposium. Purdue University, IN

  Oral Presentation: Biology of Intracellular Hepatitis B e Antigen
- 2017 International HBV meeting. The Molecular Biology of Hepatitis B Viruses. Washington D.C., United States

  Oral Presentation: Understanding Intracellular Hepatitis B e Antigen
- 2016 International HBV meeting. The Molecular Biology of Hepatitis B Viruses. Seoul, South Korea

  Oral Presentation: Biological Functions of Intracellular Hepatitis B e Antigen
- 2016-2018 IBMG Campus Visits, Indianapolis

  Poster Presentation: Biological Functions of Intracellular Hepatitis B e Antigen
- 2015 Indiana CTSI Annual Meeting, Indianapolis
- 2014, 4th International Conference on Ecotoxicology and Environmental Sciences (ICEES), New Delhi, India

  Oral Presentation: Potential bacterial bioremediation from mixed chemical slag
- 2013, 100th Indian Science Congress, Kolkata, India

Poster Presentation: Chromium uptake by bacteria isolated from *Lemna* rhizosphere in a lentic ecosystem

- 2012, 99thth Indian Science Congress, Bhubaneswar, India

  Oral Presentation: Bioaccumulation of chromium by aquatic hydrophyte *Lemna* sp. and its associated rhizosphere bacteria

- 2012, Annual College Seminar, Kolkata, India

  Oral Presentation: Angiogenesis – Jab To Grow Blood Vessels

Awards

Academic

- 2019 IUPUI Elite 50 Premier 10
- 2019 Honorable Mention Erica Daniel Kepner Award for Scientific Achievement
- 2018 International HBV Meeting Travel Grant
- 2018 EASL International Liver Congress Travel Grant
- 2017 IUSM Graduate Student Travel Award
- 2017 Graduate and Professional Educational Grant
- 2017 Cagiantas Scholarship
- 2017 International HBV Meeting Travel grant
- 2017 Merilyn Hester Scholarship Recipient
- 2016 Honorable Mention at Sigma Xi competition
- 2016 International HBV Meeting Travel grant
- 2016 Graduate and Professional Educational Grant
- 2015 IUSM PhD Fellowship

- 2011 Ira Ganguly Memorial Award, St. Xavier's College, India

Professional Recognitions

- Toastmasters International Advanced Communication Silver 2019
- Invited Speaker at IUPUI Research Day 2018
- Invited Speaker 2018 at United States- India Education Foundation, Kolkata, India
- Toastmasters International Advanced Communication Silver 2017
- Toastmasters International Advanced Leadership Bronze 2017
- Toastmasters International Competent Leadership Award 2017
- Toastmasters International Advanced Communication Bronze Award 2017
- Toastmasters International Competent Communication Award 2016

Teaching and Mentoring Experience

- Spring 2018, Mentored first year graduate student during rotation
- Summer 2017, Mentored summer student from Carmel High School, Indianapolis.
- Spring 2017, Mentored first year graduate student during rotation
- Fall 2016, Mentored first year graduate student during rotation
- Fall 2016, Teaching Assistant MICR-J 210 Microbiology and Immunology for Nursing Students.
- Summer 2016, Mentored summer student from Carmel High School, Indianapolis.
- Summer 2015, Mentored Project SEED fellow.
- 2012-2013, Part of Government of India UGC sponsored Educational Multimedia Research Centre (EMMRC) that is committed to the goal of building a bridge

between eminent resource persons based in the cities and students from far flung areas of the country by telecasting and uploading lectures-on-demand through the multicast facility of Edusat platform.

Scientific Skill Set

- Vector designing and cloning, Site-directed mutagenesis
- Western immunoblotting
- PCR (Real-Time ~, RT- ~, *etc*.).
- Southern Blot DNA hybridization
- Northern Blot RNA hybridization
- Chromatin Immunoprecipitation (ChIP)
- Luciferase assay of promotors activity, ELISA spectrophotometry (BioRad)
- Co-Immunoprecipitation of proteins
- Orthotopic implantation of cancer cells in mouse
- Cell culture- cultivations, passaging and generating stable cell lines
- Bacterial and phage culture techniques
- Agarose and Polyacrylamide Gel Electrophoresis
- Bacterial Transformation
- Viral Transduction and Infection Assays
- Confocal Microscopy and Imaging
- Gene Cloning
- Primer Designing
- Stable cell line development

- Flow Cytometry (basic experience)

Professional Activities

- 2018-2019 President at Communicators Club at IUPUI Toastmasters
- 2018 Invited Speaker at IUPUI Research Day 2018
- 2018 Invited Speaker at United States- India Education Foundation, Kolkata, India
- 2017-2018, Chair of IUSM Annual Fong-Clontech Awards Committee, Department of Microbiology and Immunology
- 2017-2018, Student Representative on IUSM IBMG Admissions Committee
- 2017-present, Graduate Student President for Department of Microbiology and Immunology at IUSM
- 2017-2018, Project Grow Pass the Torch to Women Mentee
- 2017, Featured on IUPUI Graduate Student Successes
- 2017, Associated with organization for Women in STEM Classroom Walk IN that aims at inspiring kids to become the future scientists
- 2017-2018 Area 35 Director for District 11 Toastmasters International
- 2017-2018, Dr. Ambar Banerjee shadow surgeon at IU North Hospital
- 2017-2018, Dr. Thomas Gardner shadow surgeon at University Hospital
- 2017- present, Volunteer at IU University Hospital
- 2017- present, Volunteer at Guardian Angel Hospice
- 2016-present, Involved with Science Central, Fort Wayne
- 2016-present, Member at Association of Women in Science

- 2016-present, Member of IUSM NetworkIN Committee that organizes various networking for IUSM graduate students
- 2016-2017, Co-chair of IUSM Annual Fong-Clontech Awards Committee, Department of Microbiology and Immunology
- 2016,"Portal to Public" program Science Fellow with Science Central, Fort Wayne that aimed explaining Science in layman terms to the general public
- 2016, Panelist at Global Voices Speaker Program
- 2015-2016, IUSM Events & Engagement Committee Member
- 2015-present, Member at Scientific Toastmasters at IUSM
- 2015-present, Student Ambassador for IBMG Campus Visits, IUSM
- 2015-present, Volunteer Mentor at IUPUI Graduate Welcome
- 2015, Graduate Student representative for the IBMG graduate Division at LSAMP recruitment event
- 2014-present, Member of Bengali Association of Indiana and involved in various cultural events including theatre, dancing, poem recitation, music
- 2012-2014, Volunteer at an NGO "Bubhaneswari Amra Sabai" as a part time teacher to students from economically disadvantaged backgrounds