Characterizing the Impact of 5' PreS1 Deletion Hepatitis B Virus Mutants on Endoplasmic Reticulum Stress

By

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Abstract

Hepatitis B Virus (HBV) remains a significant global health burden, chronically infecting over 350 million individuals worldwide and increasing the risk of severe liver complications, including hepatocellular carcinoma (HCC) and liver cirrhosis. Additionally, chronic infection of HBV can lead to an accumulation of mutations within the viral genome, some of which have been implicated in disease progression. Notably, in-frame deletions in the preS1 region of the envelope gene have been associated with increased viral infectivity and pathogenicity.

In this study, we investigated the effects of a 5' preS1 deletion mutant of genotype C origin on ER stress in hepatocytes, which is a common feature prior to cancer development. We specifically examined the expression levels of key ER stress responsible genes, including GRP78, GADD153, and ATF6, to determine whether the mutant induces a more pronounced ER stress response compared to the wild-type virus in hepatoma cell lines Huh7 and HepG2 after co-transfection of the reporter gene with HBV DNA. Additionally, HBsAg production was assessed as an indicator of viral replication and protein expression of the mutant, providing insight into how preS1 mutations may contribute to HBV persistence and pathogenicity. Lastly, the clinical relevance of the 5' preS1 mutant was assessed by measuring the ER stress levels in chronically infected liver cells in vitro.

By elucidating the mechanisms by which preS1 deletions contribute to liver injury, this study provides new insights into the molecular underpinnings of HBV-induced liver disease. A deeper understanding of these processes is crucial for developing targeted therapeutic interventions, ultimately reducing the public health burden associated with chronic HBV infection.

Chapter 1: Introduction

Epidemiology

Hepatitis B Virus (HBV) continues to pose a large global health crisis, with approximately 1.5 million people being newly diagnosed each year[1]. HBV is responsible for roughly 820,000 deaths annually, primarily due to severe liver complications[2]. The virus is primarily transmitted through bodily fluids such as blood, saliva, semen, and vaginal secretions, with perinatal mother-to-child transmission (vertical transmission) being the most common route[3].

HBV disproportionately affects individuals residing in developing regions of the world, particularly Sub-Saharan Africa and East Asia[4]. Despite the availability of a highly effective vaccine introduced in the 1980s, limited healthcare infrastructure and socioeconomic barriers—such as the lack of public health funding and access to antiviral medications—continue to fuel the global burden of both acute and chronic HBV. The vaccination of infants at birth would serve as a safeguard against HBV infection; however, in 2022, only 46% of infants worldwide received the birth dose of the Hepatitis B virus vaccine[5].

In healthy adults, 95% of HBV infections are cleared without medical intervention[9]. If the infection develops into a chronic state, severe liver complications such as liver cirrhosis and hepatocellular carcinoma (HCC) can accompany this progression[6]. Chronic HBV infection is the leading cause of HCC as it is associated with over 70% of HCC cases[7].
Additionally, HBV mutants associated with chronic infection can increase infectivity and viral replication, which have also been shown to exacerbate disease progression and challenge patient treatment as well as disease prevention[26].

Structure and Genome Organization

HBV belongs to the *Hepadnaviridae* family and possesses unique structural and genomic characteristics that distinguish it from other viruses. Its genome features a relaxed, circular DNA (rcDNA) that is partially double-stranded and spans approximately 3.2kb in length – making it the smallest genome of any DNA virus[4,8]. The genome is encapsulated within a core particle, which is further surrounded by an envelope containing surface proteins.

Once the virus enters the host cell, the rcDNA is repaired in the nucleus and converted into a covalently closed circular DNA (cccDNA), a stable episomal form that serves as the transcriptional template for viral protein expression and viral DNA replication. The HBV genome comprises four overlapping open reading frames (ORF): P, preS/S, preC/C, and X[8,9] (Fig. 1A).

The P ORF encodes the viral polymerase, also known as reverse transcriptase, which is essential for viral replication through reverse transcription of the pregenomic RNA[8,9]. The preS/S ORF encodes the envelope proteins, with alternative translation initiation generating three forms of surface antigens (HBsAg): small (S-HBs), middle (M-HBs), and large (L-HBs)[8,9] (Fig. 1B) The preC/C ORF produces the core protein, which forms the viral nucleocapsid, as well as HBeAg, a secreted protein thought to modulate immune response[8]. Lastly, the X ORF encodes the HBx protein, a multifunctional regulatory protein implicated in viral replication and pathogenesis[8].

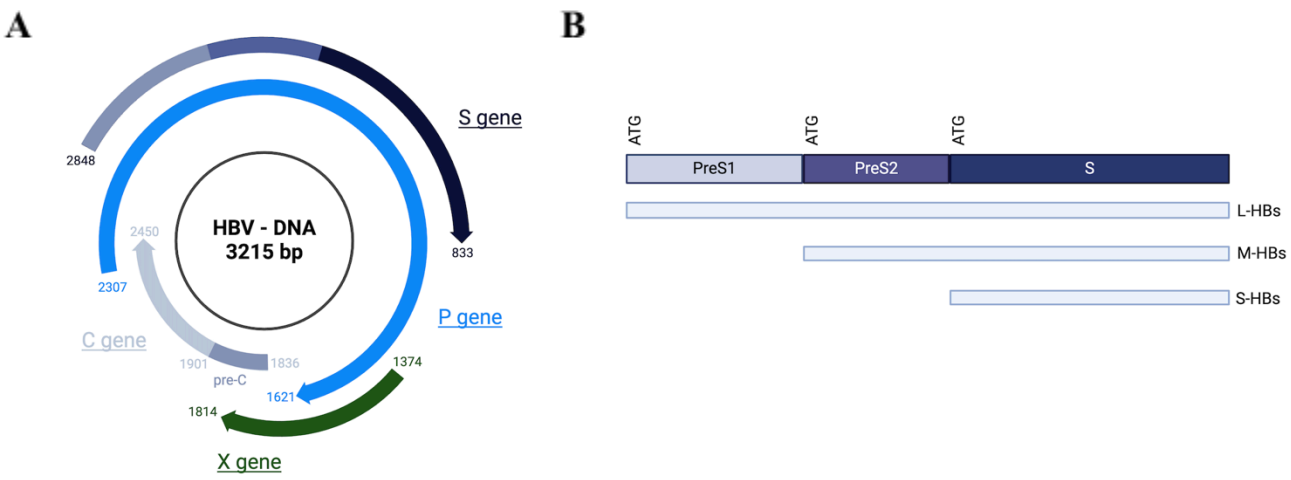

Figure 1. Hepatitis B Virus genome (A). The envelope gene consists of three in-frame start codons that subdivide the gene into preS1, preS2, and S regions, enabling the expression of three envelope proteins: L, M, and S protein (B).

Hepatitis B Virus Life Cycle

Hepatitis B virus is hepatotropic, meaning it primarily infects the liver and its residing hepatocytes. HBV gains access to hepatocytes by first binding non-specifically to factors such as heparan sulfate proteoglycans (HSPGs) with low affinity then binding with more specificity and higher affinity to sodium taurocholate co-transporting peptide (NTCP), a liver specific receptor[10] (Fig. 2). After binding to two surface receptors of hepatocytes, it is widely hypothesized that the virus enters the host cell in an endocytosis-mediated manner[10].

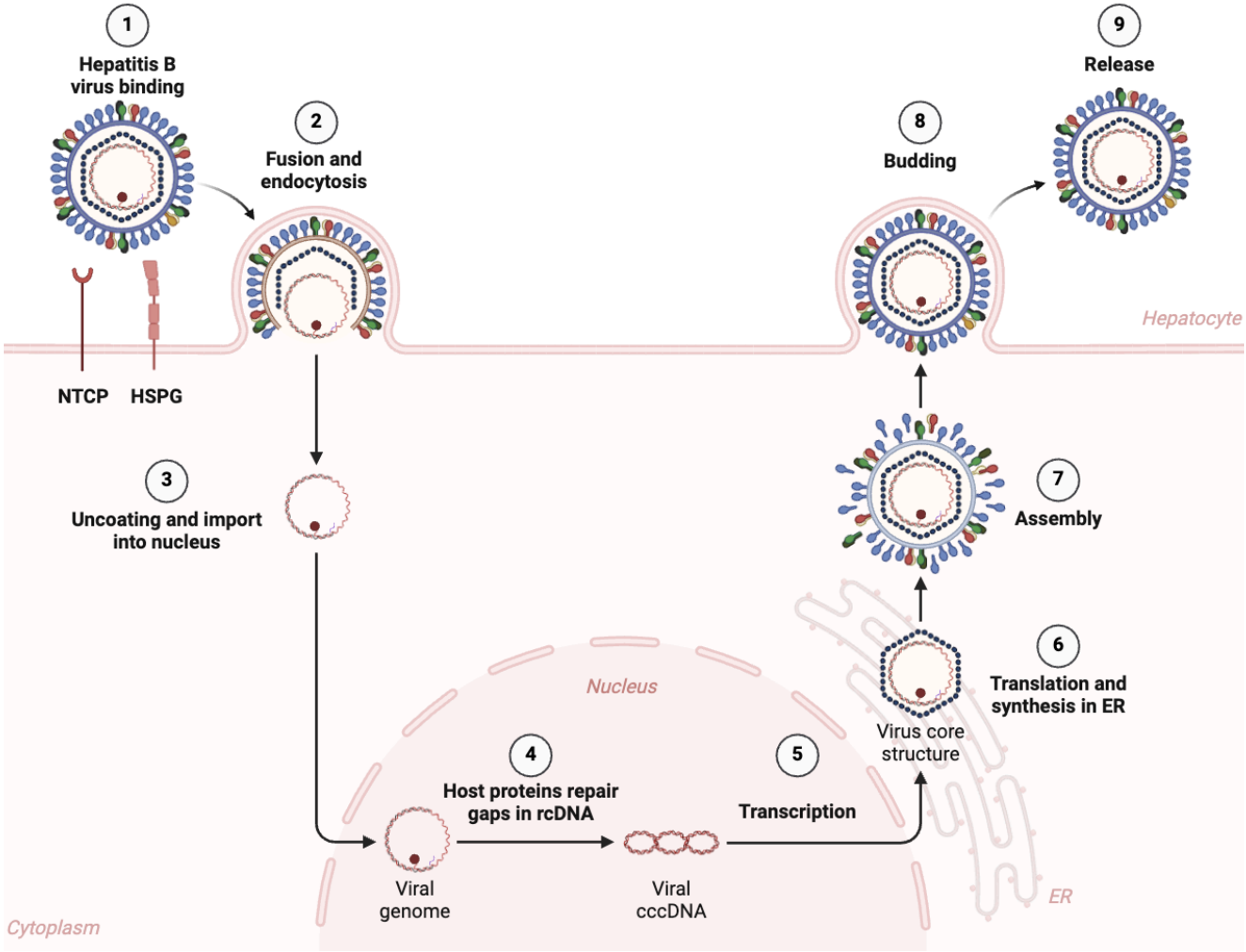

Figure 2. Diagram of Hepatitis B Virus life cycle. HBV will bind to both HSPG and NTCP, allowing it to become endocytosed into the host cell. In the host cell, the viral rcDNA will make its way to the nucleus where it will use host machinery to repair to cccDNA, using this as a template for transcription. Viral protein is synthesized in the endoplasmic reticulum (ER), assembled, and released. Figure created using BioRender.

The viral envelope fuses with the endosomal membrane in which the nucleocapsid is released into the cytoplasm[8]. The nucleocapsid will then uncoat and the viral DNA will eventually make its way to the nucleus via classical nuclear localization signals (NLS)[8,9]. In the nucleus, the rcDNA is repaired and converted into cccDNA using host machinery, which serves as a more stable template for transcription. The cccDNA is transcribed into four varying lengths of RNA: 3.5kb, 2.4kb, 2.1kb and 0.7kb. There are two approximately 3.5kb RNA transcripts: the

longer pre-core RNA (pcRNA) and the shorter pre-genomic RNA (pgRNA)[4,10]. The pcRNA transcript will result in HBeAg while the pgRNA will translate into the core and polymerase protein product[4,10]. The 2.4kb RNA produces L-HBs while the 2.1kb RNA produces M-HBs and S-HBs[4,10]. Lastly, the 0.7kb RNA produces HBx[4,10].

Viral RNA is then transported to the cytoplasm, where it can be translated into seven different viral proteins. Furthermore, the pgRNA is packaged into core particles with HBV polymerase, which reverse transcribes the pgRNA back into rcDNA[9,10]. These core particles, in which the reverse transcription occurs, are either enveloped for release as virions or it is transported back to the nucleus for further cccDNA formation[9,10].

Endoplasmic Reticulum (ER) Stress and HBV

Hepatocytes have a highly functional endoplasmic reticulum (ER) as the liver is responsible for synthesizing large amounts of proteins[12]. The ER is an organelle within eukaryotic cells that play a large role in maintaining protein homeostasis by synthesizing, folding, and modifying proteins[6]. The organelle furthermore senses misfolded or unfolded proteins and prohibits their exit out of the ER, allowing only normal proteins to pass through to the Golgi apparatus[12]. ER stress can be triggered by several different stimuli, including a viral infection, which causes an accumulation of misfolded protein. ER stress is sensed by three transmembrane proteins: IRE1, PERK, and ATF6[13] (Fig. 3). These transmembrane proteins are initially bound to GRP78, an important ER chaperone that is involved in facilitating protein folding, targeting misfolded proteins for degradation, and transporting nascent proteins across the ER membrane[14,15,16].

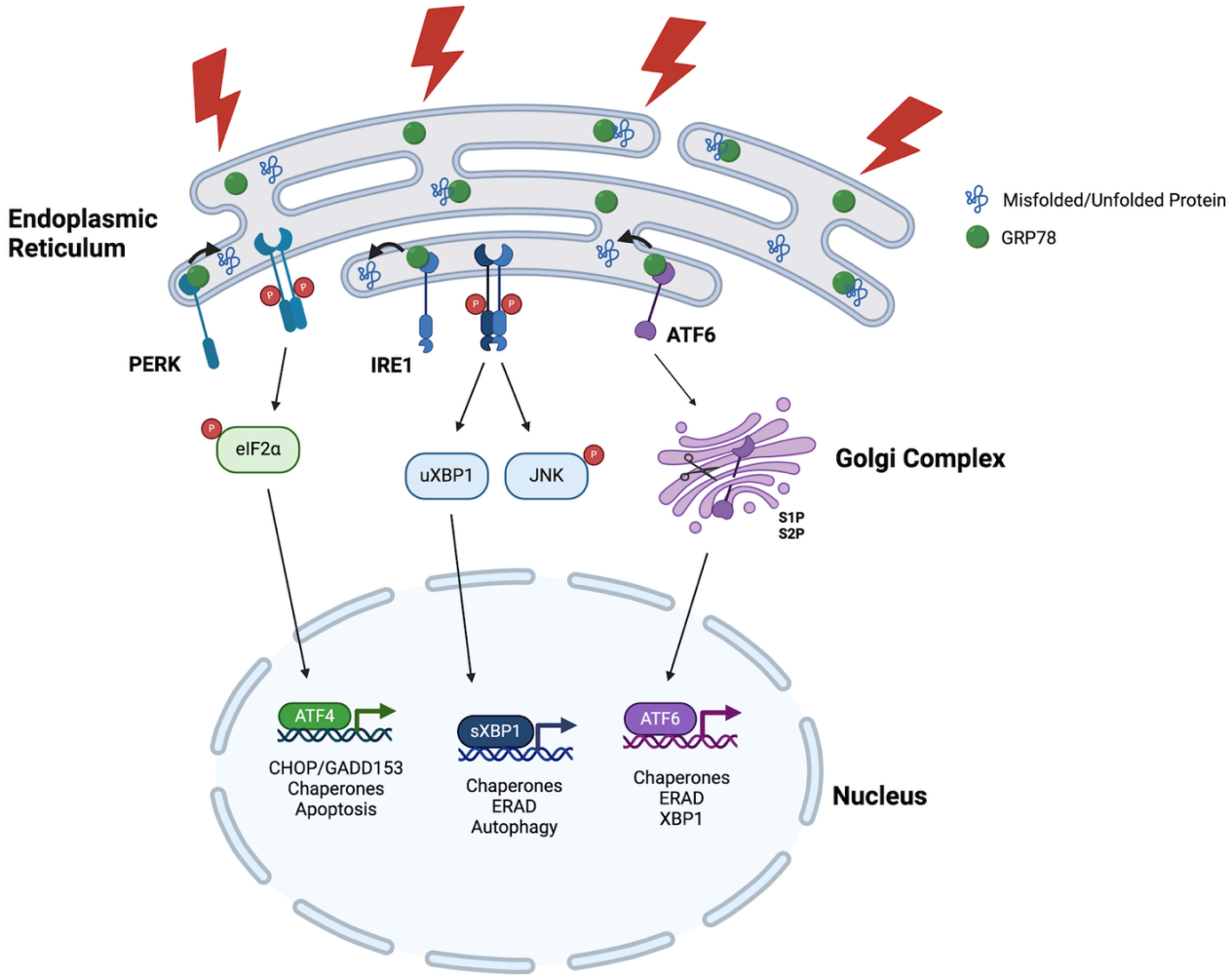

Figure 3. Diagram of the unfolded protein response (UPR). Figure created using BioRender.

In response to an accumulation of misfolded protein, GRP78 will disengage with the transmembrane proteins and preferentially bind to the misfolded proteins, subsequently triggering the cascade of events that make up the unfolded protein response (UPR)[12,17,18]. The UPR aims to alleviate ER stress and restore protein homeostasis through various mechanisms, including the inhibition of protein synthesis, the upregulation of enzymes involved in protein degradation, and the upregulation of ER chaperones[12,17,18].

Several molecular markers can be used to monitor ER stress, including GRP78, ATF6, and GADD153. GRP78, in particular, is transcriptionally upregulated during ER stress and

serves as a key indicator of UPR activation[20,21]. ATF6, another critical component, initially resides as a transmembrane protein within the ER, bound to GRP78 during normal conditions[22,23]. Upon ER stress, ATF6 dissociates from GRP78 and translocates to the Golgi complex for proteolytic processing, converting into an active transcription factor that upregulates ER chaperones, including GRP78[24,25].

In cases of prolonged or severe ER stress, such as that observed in chronic HBV infection, the UPR may shift from protective to pro-apoptotic signaling[16]. One such pro-apoptotic marker is GADD153 (also known as CHOP), which is induced under sustained ER stress conditions and promotes cell death pathways[26]. The ability of HBV to manipulate ER stress pathways, particularly through the regulation of GRP78 and ATF6, may contribute to viral pathogenesis and hepatocyte apoptosis. Further examination of these molecular markers in the context of HBV infection, especially infection with mutants with increased replication and infectivity capacity will provide insight into how ER stress contributes to severe liver disease progression.

Deletions in the PreS1 Region

The compact nature of the HBV genome gives rise to pleiotropic effects caused by mutations, which can include various parts of the genome. There is a unique mutation that spans the 5' end of the preS1 region and is either 15, 18, or 21-nucleotides (nts) in length. This mutation furthermore affects the preS1 ATG codon and results in a truncated preS1 domain in which the P protein is shortened by 5- or 6-amino acids and the L protein is shortened by 11-amino acids[27]. The shortened L protein, a crucial component of the HBV envelope, allows for more efficient viral infection, through its increased binding affinity to the NTCP receptor[27]. In

addition, these naturally occurring deletions within the preS1 gene of HBV genotype B and genotype C have been found to increase viral replication.

Furthermore, the preS1 start codon deletion has been shown to be highly prevalent in chronic HBV patients with genotype C[27,28]. These deletions have also been identified in patients with elevated levels of hepatitis B surface antigen (HBsAg) and high HBV DNA titers[27]. Despite the increased levels of viral markers, patients exhibited stable alanine aminotransferase (ALT) levels, a key indicator of liver damage[27]. However, liver biopsies revealed advanced liver fibrosis, suggesting that significant disease progression can occur even in the absence of ALT elevation.

Given that ALT is a conventional marker of liver damage but remained unchanged in these patients, other pathological mechanisms – such as ER stress and oxidative stress – may be at play. This aligns with the focus of the present study, which investigates whether the preS1 mutant induces ER stress responses and contributes to liver injury through mechanisms not reflected in ALT fluctuations, such as intracellular pathological changes as opposed to cell death. Persistent intracellular abnormality may result in genetic alterations associated with cancer development. Understanding how preS1 deletions influence HBV-induced liver pathology is therefore critical for identifying novel therapeutic targets aimed at mitigating liver damage in affected patients.

These findings suggest that the presence of 5' preS1 deletions may contribute to disease progression through mechanisms beyond direct hepatocyte death. The implications of such mutations are thought to include increased ER stress, immune escape, and alterations in viral protein production – factors that may collectively exacerbate liver injury. As chronic HBV

infection persists, selective pressures favoring these mutations could further drive disease progression.

Significance of the Study

Chronic HBV currently affects 350-400 million individuals worldwide and significantly increases the risk of severe liver diseases[3,29]. Chronic infection is also associated with the emergence of mutations within the HBV genome, including the 18-nt preS1 deletion examined in this thesis[9]. The molecular mechanisms by which HBV mutants contribute to pathogenesis remain unclear, highlighting the need for further investigation into their role in disease progression. One emerging area of interest is ER stress, which has been implicated in hepatocyte dysfunction and liver pathology.

Among the various HBV mutations that arise during chronic infection, deletions in the preS1 region of the envelope gene have been linked to increased disease severity[27,28]. Prior studies suggest that these mutations may contribute to hepatocyte injury by inducing ER stress, yet the precise impact of 5' preS1 deletions on ER stress pathways remains unclear[27,28]. By identifying the molecular consequences of preS1 deletions, this study contributes to a deeper meaning of understanding of HBV-induced liver disease. These findings may provide a foundation for developing targeted therapeutic strategies aimed at mitigating ER stress-associated liver damage in chronic HBV patients. By uncovering the molecular mechanisms through which HBV mutants induce ER stress, further identification of potential therapeutic targets can be made to restore cellular balance and reduce hepatocyte dysfunction. Understanding this relationship is essential for elucidating the role of viral mutations in HBV pathogenesis.

Chapter 2: Materials and Methods

Cell Culture

HuH7 cells derived from a hepatoma cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin. HepG2 cells derived from another hepatoma cell line were cultured in Eagle Minimum Essential Medium (EMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured in either 6- or 12-well formats to 60-70% confluency and transfected using a polyamine-based transfection reagent (Mirus). All cells were grown at 37°C in a 5% CO_2 incubator.

Promoter Activity Assay

Plasmids containing the promoters of GRP78, ATF6, and GADD153 cloned into a promoter-less luciferase reporter backbone were obtained from prior work[30]. These pre-constructed plasmids, along with 1.1mer replication competent form of HBV DNA constructs of a genotype C clone, were co-transfected into Huh7 or HepG2 cells using polyamine, following the manufacturer's protocol. The HBV DNA constructs used were either wildtype (55.3) or the mutant (55.3-18nt).

Cells were harvested at 72 to 96 hours post-transfection and lysed using a luciferase-based cell lysis buffer (Promega), ensuring complete lysis by gentle agitation incubation on ice for ten minutes. The lysates were clarified by brief centrifugation to remove cellular debris. Luciferase activity was measured using a luciferase assay kit (Promega).

Detection of HBsAg by enzyme linked immunosorbent assay (ELISA)

Intracellular and extracellular HBsAg levels were quantified using commercially available ELISA kits, following the manufacturer's protocol (KHB Inc). For intracellular detection, cell lysates were prepared by lysing the cells with 0.5% NP40/20mM Hepes, pH7.4 followed by centrifugation to remove cellular debris. For extracellular HBsAg, cell culture supernatants were collected, clarified by centrifugation if necessary, and directly used in the assay. Samples were added to the pre-coated wells, incubated with detection antibodies, and processed as per the kit instructions, including washing steps between reagent additions. The chromogenic substrate was then added, and the reaction was stopped with the kit-included stop solution. Absorbance was measured at 450 nm with a reference wavelength of 630 nm using a microplate reader to correct for background signal.

To ensure accurate quantification, samples with optical density (OD) readings exceeding the linear range of the assay (OD > 2.5) were diluted. These diluted samples were re-measured to avoid signal saturation and ensure reliable data.

Statistical Analysis

The difference between the 5' preS1 deletion mutant and the wild type were analyzed using a Student's t-test where a p-value of <0.05 is considered to be statistically significant.

Chapter 3: Results

Transfection of Mutant HBV DNA increases HBsAg production in transfected cells.

To investigate the impact of the 5' preS1 deletion on HBsAg production, Huh7 cells were transfected with either wildtype or mutant HBV DNA constructs. Four days post-transfection, cell lysate and media were collected for intracellular and extracellular HBsAg detection by ELISA. HBsAg serves as a critical biomarker for active HBV replication and envelope protein expression, a major viral protein processed via ER[31].

HBsAg levels were higher in samples transfected with the 5' preS1 deletion mutant compared to those transfected with the wild type HBV DNA (Fig. 4). This observation suggests that the 5' preS1 deletion enhances HBsAg production, potentially due to increased viral infectivity or alteration in viral protein expression. These results align with previous studies linking preS1 deletions to heightened pathogenicity and may indicate a role for the preS1 region in modulating viral gene expression and infectivity.

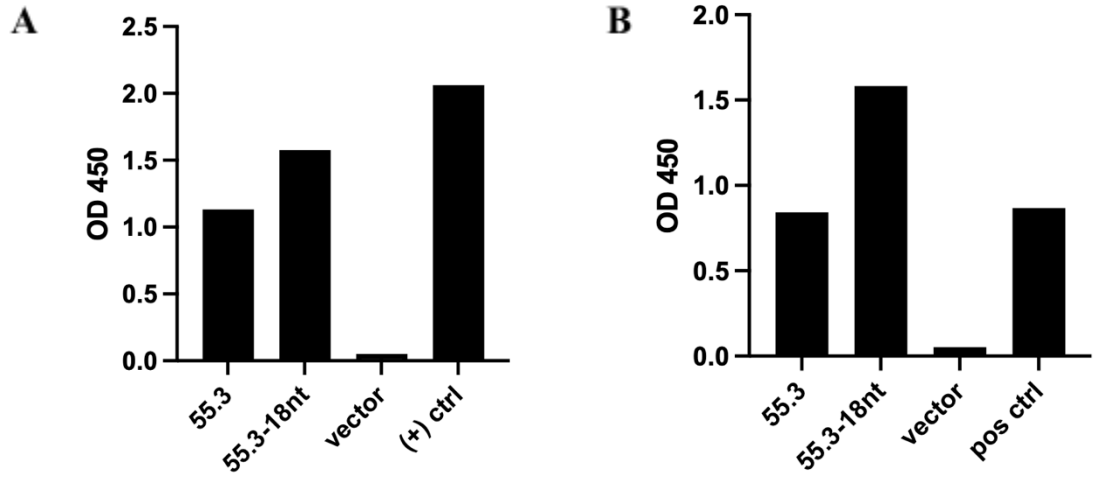

Figure 4. HBsAg levels of Huh7 cells co-transfected with HBV DNA and a GRP78 promoter construct. Intracellular (A) and extracellular (B) HBsAg levels were measured from cell lysates and media, respectively, using ELISA. A positive control provided with the kit was included as a reference. Due to limitations in time and materials, the assay was performed once; therefore, statistical analysis was not conducted.

Mutant HBV DNA induces elevated ER stress in Huh7 and HepG2 cells. To test whether the 5' preS1 deletion mutant impacted ER stress levels, both Huh7 and HepG2 cells were co-transfected with wild type or mutant HBV DNA and an ER stress promoter construct: GRP78, GADD153, or ATF6. Luciferase activity, which signifies the promoter activity of the ER stress response genes, was measured in cell lysate four days post-transfection (Fig. 5A-D). Cells co-transfected HBV DNA and GRP78 exhibited a significant increase in luciferase activity with the 18nt deletion mutant compared to the wild type (Fig. 5A). Furthermore, the increase in GRP78 levels between the mutant and wild-type was more pronounced than the increase in GADD153 and ATF6 levels between the mutant and wild-type.

HepG2 cells, another human hepatoma cell lines, was utilized alongside Huh7 cells to further validate the promoter activity of GRP78. HepG2 cells were co-transfected with either wildtype or mutant HBV DNA and the GRP78 promoter construct to assess whether the observed trend in GRP78 promoter activity was reproducible across different hepatic cell models. The resulting data demonstrated a similar pattern in GRP78 promoter activity in HepG2 cells as previously observed in Huh7 cells, with consistent differences seen between the wildtype and mutant HBV samples (Fig. 5D). This cross-validation across two hepatoma cell lines strengthens the reliability of the findings and suggests that the regulation of GRP78 promoter activity by HBV is not limited to a single cell line but may reflect a broader biological response in hepatic contexts.

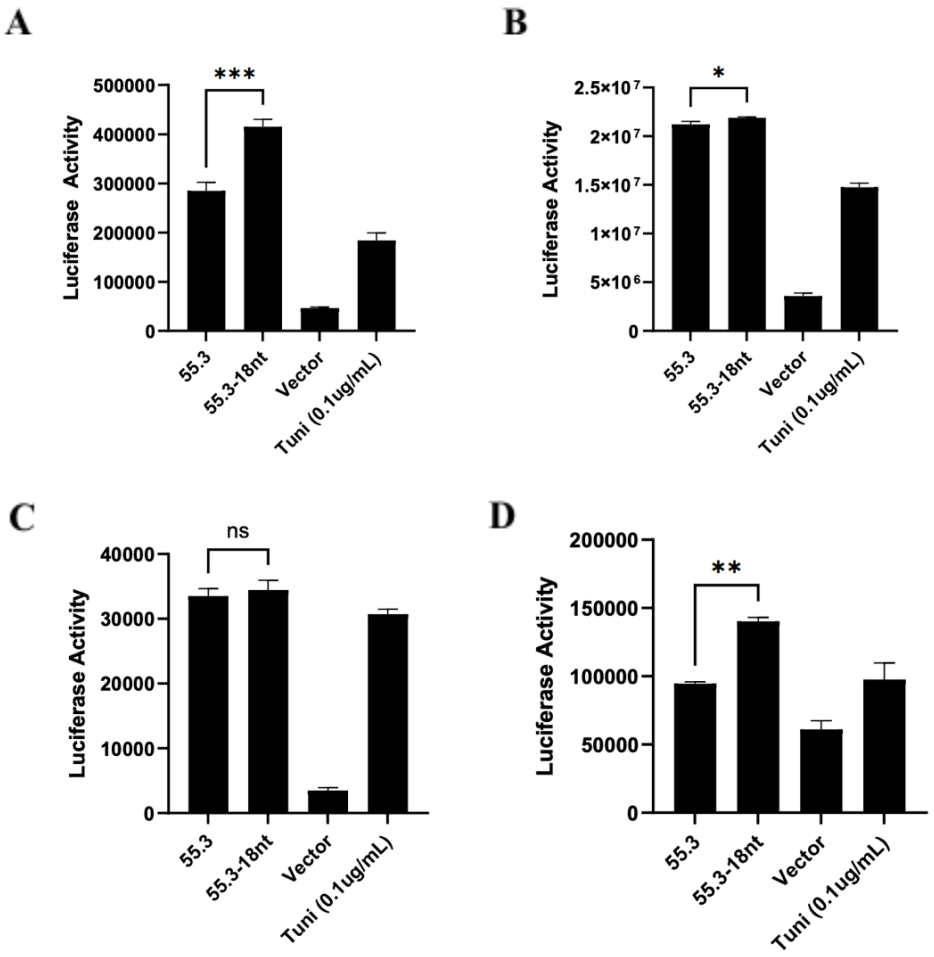

Figure 5. Luciferase activity in cells co-transfected with HBV DNA and ER stress promoter constructs, measured using promoter activity assay. Huh7 cells co-transfected with HBV DNA and GRP78, GADD153, and ATF6 promoter constructs (A-C, respectively). HepG2 cells co-transfected with HBV DNA and GRP78 (D). Significance was assessed using one way t-test, *p<0.05; **p<0.01; ***p<0.001.

Chronic infection induced by the 5' preS1 HBV mutant elevates ER stress.

Alternatively, we used an in vitro HBV infection system to assess the impact of chronic HBV infection on ER stress, Huh7 cells were transfected with either wildtype or mutant HBV DNA. To generate infectious HBV particles, media was collected on days three and five post-transfection, combined, and subjected to purification and viral pelleting (Fig. 6A). The

resulting viral pellet was resuspended with FBS (-) MEM containing 4% PEG 8000 and used to infect HepG2/NTCP cells, a hepatocyte cell line engineered to express sodium taurocholate co-transporting polypeptide (NTCP), rendering it permissive to HBV infection.

After 25 days post-infection, which mimics chronic HBV infection, cells were transfected with a GRP78 promoter construct. Four days post-transfection, cells were harvested, and the cell lysates were subjected to a promoter activity assay to measure ER stress levels. The prolonged presence of HBV resulted in a significant increase in promoter activity in mutant samples compared to the wildtype (Fig. 6B). These findings suggest that the 5' preS1 deletion mutant may exacerbated ER stress during chronic HBV infection, possibly due to overexpression of the viral proteins to increase the ER burden.

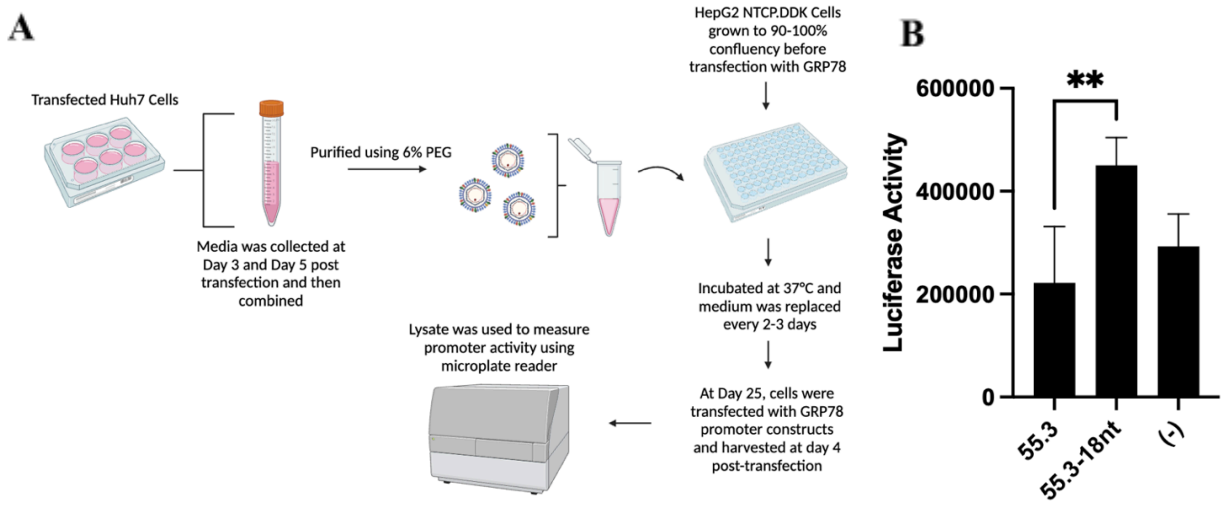

Figure 6. Chronic infection of HepG2/NTCP cells with the preS1 mutant. A diagram illustrating the infection process of HepG2/NTCP cells: media from Huh7 cells transfected with HBV DNA were collected on days 3 and 5, combined, purified to extract the virus, and then used to infect HepG2/NTCP cells (A). Luciferase activity of lysate was measured using a luciferase driven promoter activity assay (B). **p<0.01; NTCP, sodium taurocholate co-transporting peptide.

Chapter 4: Discussion

HBV-induced chronic infection plays a significant role in causing severe liver complications among patients with 1.5 million new infections diagnosed each year[1]. While the mechanisms underlying HBV persistence and liver disease progression remain incompletely understood, it is established that ER stress has been implicated as a critical factor. In this present study, we characterized the ER stress response induced by a 5' preS1 deletion mutant of increased infectivity and replication and examined its impact on key molecular markers of the UPR. Clinical evidence shows that the 5' deletion mutant is associated with earlier development of fibrosis[27]. This study investigated whether the higher-replicating HBV variant is associated with increased ER stress, which has a potential to advance disease progression.

The lysate and media of mutant-transfected cells were also used to examine HBsAg levels, which serve as a marker for the level of viral replication and envelope protein expression. The results indicated that 5' preS1 mutant samples exhibited higher levels of HBsAg in comparison to the wildtype samples, aligning with previous findings that demonstrated increased viral replication and infectivity associated with this mutant[27]. Based on current understanding of HBV biology and mechanisms of infection, it can be hypothesized that the 5' preS1 mutation may enhance HBV surface protein expression or stability, contributing to the elevated HBsAg levels in both the intracellular and extracellular environments. This is consistent with the fact that HBV is not a cytolytic virus, but rather causes chronic intracellular injury with the potential to impair cell homeostasis leading to liver disease or cancer.

The present findings demonstrate that the 5' preS1 mutants induced higher levels of ER stress in comparison to the wildtype counterpart, with significant increases observed in cells transfected with GRP78. ER stress levels mediated by GADD153 and ATF6 were observed to

have insignificant changes between the mutant and wildtype samples. A greater difference of GRP78 levels was observed between mutant and wildtype samples, compared to GADD153 and ATF6 levels between the two samples, signifying that mutant-induced ER stress promotes GRP78 transcription as an initial response during infection. This can also be due to the fact that GRP78 is a key regulator of the UPR and plays a role in all three ER stress pathways, whereas GADD153 and ATF6 each play a role in a singular pathway. Additionally, GADD153 is most associated with apoptosis, which is reserved for severe and prolonged cases of ER stress.

To further confirm these findings, GRP78 mediated expression was assessed in an additional hepatocytic cell line, HepG2. A similar trend was observed, in which there were higher levels of ER stress in cells transfected with the mutant compared to those transfected with the wildtype virus. This consistency across multiple cell lines further supports the hypothesis that the 5' preS1 mutant exacerbates ER stress.

Lastly, we validated the observations from HBV transfected cells in HBV infection cell system, which is more physiologically relevant to in vivo infection. HepG2/NTCP cells were chronically infected with virus produced by mutant and wild-type HBV, which were then subsequently transfected with GRP78 to examine ER stress levels. It was, again, found that GRP78 levels were higher in mutant samples compared to the wildtype samples. These findings solidify the idea that 5' preS1 mutants exacerbate ER stress levels compared to the wildtype virus in clinically relevant samples. Additionally, this adds to the importance of why this should be studied because it has this profound impact on chronically infected cells, which translate to chronic infection in patients and the underlying disease pathology.

As previously mentioned, this mutant has been detected in patients with high HBsAg and HBV DNA titers yet persistently normal ALT, despite liver biopsy findings of advanced

fibrosis[27]. This discrepancy suggests that ALT alone may not fully reflect the extent of liver damage in these patients. Given our findings that this mutant induces elevated ER stress, it is possible that this molecular mechanism can contribute to liver fibrosis progression. Future studies, including ROS production, should explore alternative biomarkers that better capture disease severity in patients harboring this mutation.

The 5' preS1 mutant leads to increased viral replication and disrupts hepatocyte function, with the present study providing compelling evidence that it also exacerbates ER stress. Given the profound impact of ER stress on chronic HBV infection, understanding the molecular mechanisms by which this mutation influences viral pathogenesis could provide valuable insights into potential therapeutic interventions aimed at mitigating HBV-induced liver disease.

Chapter 5: Conclusion and Future Directions

There is still much left to explore regarding how the 5' preS1 mutant influences HBV pathogenicity, with ER stress being just one aspect of its large impact. Further investigation into the specific signaling pathways involved in ER stress and their temporal dynamics could provide crucial insights into the molecular mechanisms driving disease progression. Identifying key regulatory axes and their activation windows may offer potential therapeutic targets for mitigating chronic HBV infections caused by this mutant.

The relationship between elevated ER stress and reactive oxygen species (ROS) levels warrants further study. Given that oxidative stress is a known contributor to liver disease, understanding how the 5' preS1 mutant exacerbates ROS production could shed light on its role in promoting liver injury and disease severity. To further investigate the consequences of increased ER stress and ROS accumulation, cell viability and death assays can be utilized to analyze the viability-to-death ratio, providing insight into the extent of hepatocyte damage. Elucidating these mechanisms could pave the way for targeted interventions aimed at reducing cellular stress and limiting liver damage in patients with chronic HBV infections associated with this mutant.

Additionally, identifying the specific viral protein that plays a major role in ER stress may be of interest. In this study, full-length, replication-competent HBV DNA was used. To determine the contribution of individual viral proteins, hepatocytes can be co-transfected with an expression plasmid encoding a single viral protein along with a plasmid expressing luciferase under control of the promoter of ER stress response reporter gene. This approach can help identify which viral protein is primarily responsible for inducing ER stress, distinguish whether ER stress is triggered directly by a specific viral protein or as a consequence of overall viral

replication, and provide insight into the molecular pathways involved. These findings could aid in pinpointing potential therapeutic targets aimed at alleviating HBV-induced ER stress and its role in disease progression.

This study highlights the potential role of the 5' preS1 mutant in modulating ER stress and its broader implications for HBV pathogenicity. The findings suggest that increased ER stress as induced by this mutant may play a critical role in pathogenicity, though the precise signaling mechanisms remain to be fully elucidated. Understanding how this mutant alters intracellular stress pathways and contributes to liver injury could provide valuable insights into HBV disease progression. Future research exploring the interplay between ER stress, oxidative stress, and HBV replication may offer new therapeutic targets for managing chronic HBV infections.

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