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**Establishing an *In Vitro* Model for CRISPR-Cas9
Screening of Interferon Effectors in HBV Infection**

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1-Abstract

Hepatitis B virus (HBV) is a hepatotropic virus that can cause both acute and chronic liver infection. According to the World Health Organization, 254 million people were living with chronic hepatitis B virus (CHB) infection in 2022, with approximately 1.2 million new cases each year. While vaccination prevents new infections, CHB still leads to cirrhosis and hepatocellular carcinoma, contributing to over one million deaths annually and underscoring the need for improved therapies (World Health Organization 2025). Interferon-alpha (IFN- α), including its pegylated forms, is approved for CHB treatment, but its efficacy is limited to a small subset of patients, and its mechanism of action remains poorly understood. Indeed, interferon therapy can activate different immune cell types, but only a fraction of CHB patients achieve durable clinical benefit, underscoring the need to better understand the molecular basis of its limited efficacy (Ye and Chen 2021). We hypothesize that specific interferon-stimulated genes (ISGs) act as key effectors of the antiviral response and sought to identify them via a CRISPR-Cas9 knockout screen targeting ~2,000 ISGs. Identifying the ISGs that mediate antiviral activity may provide insights into how interferon responses can be potentiated, both to improve patient outcomes and to increase our understanding of their mechanism of action. In parallel, we aim to determine, in the context of HBV infection, which interferon subtype elicits the most robust *in vitro* response, thereby providing the most suitable condition for the screening.

A successful genetic screen requires an *in vitro* model that supports efficient HBV infection and retains intact IFN responsiveness. Since no such model was readily available, we systematically evaluated candidate cellular models and identified HepG2-NTCP cells cultured with 2.5% DMSO as optimal. This condition promotes hepatocyte-like differentiation and supports both robust HBV infection and strong response to IFN- α .

In addition to infection and IFN sensitivity, effective screening requires a reliable method to detect and enrich HBV-infected cells. After evaluating a reporter virus approach, we developed a robust single-cell detection method based on intracellular staining of HBV core antigen (HBcAg).

In conclusion, our study establishes a reliable experimental system for genetic screening of IFN effectors in HBV infection and provides a biologically relevant system for dissecting the antiviral mechanisms of interferon against HBV.

1-Riassunto

Il virus dell'epatite B (HBV) è un virus epatotropico in grado di causare infezioni epatiche sia acute che croniche. Secondo l'Organizzazione Mondiale della Sanità, nel 2022 circa 254 milioni di persone vivevano con un'infezione cronica da HBV (CHB), con circa 1,2 milioni di nuovi casi ogni anno. Sebbene la vaccinazione prevenga le nuove infezioni, la CHB continua a determinare cirrosi ed epatocarcinoma, contribuendo a oltre un milione di decessi annuali and sottolineando l'urgenza di terapie più efficaci (World Health Organization 2025). L'interferone-alfa (IFN- α), comprese le sue forme pegilate, è approvato per il trattamento della CHB, ma la sua efficacia è limitata a una piccola frazione di pazienti and il suo meccanismo d'azione rimane poco chiarito. Infatti, sebbene la terapia con interferone possa attivare diversi tipi di cellule immunitarie, solo una parte dei pazienti con CHB ottiene un beneficio clinico duraturo, evidenziando la necessità di comprendere meglio le basi molecolari della sua efficacia limitata (Ye and Chen 2021).

Abbiamo ipotizzato che specifici geni stimolati dall'interferone (ISG) agiscano come principali effettori della risposta antivirale and abbiamo cercato di identificarli tramite uno screening di knockout basato su CRISPR-Cas9 di circa 2.000 ISG. L'identificazione degli ISG che mediano l'attività antivirale può fornire nuove conoscenze su come potenziare le risposte all'interferone, sia per migliorare gli esiti clinici sia per chiarire i meccanismi alla base della sua azione. Parallelamente, miriamo a determinare, nel contesto dell'infezione da HBV, quale sottotipo di interferone induca la risposta in vitro più robusta, individuando così la condizione più idonea per lo screening.

Uno screening genetico efficace richiede un modello in vitro capace di supportare un'infezione da HBV efficiente and di mantenere una risposta integra all'IFN. Poiché non era disponibile un modello adeguato, abbiamo valutato diversi sistemi cellulari and identificato le cellule HepG2-NTCP coltivate con 2,5% di DMSO come ottimali, in grado di sostenere sia un'infezione efficiente da HBV sia una robusta risposta a IFN- α . Inoltre, per rilevare in modo affidabile le cellule infettate, abbiamo sviluppato un metodo di analisi a singola cellula basato sullo staining intracellulare dell'antigene core di HBV (HBcAg). In conclusione, il nostro studio fornisce un sistema sperimentale solido per lo screening genetico degli effettori dell'interferone and per lo studio dei meccanismi antivirali contro HBV.

2-Introduction

2.1- HBV

According to the World Health Organization, nearly one-third of the global population has been infected with hepatitis B virus (HBV) at some point in their lives. While most individuals acquire the virus in adulthood and experience a self-limiting acute hepatitis B (AHB), fewer than 5% of immunocompetent adults progress to chronic hepatitis B (CHB). In contrast, infections acquired during infancy or early childhood have a much higher likelihood of becoming chronic. Chronic HBV infection affects over 250 million people worldwide and is responsible for nearly one million deaths annually, primarily due to complications such as liver cirrhosis and hepatocellular carcinoma (HCC) (Revill et al. 2019) (Yuen et al. 2018a). Hepatitis B virus (HBV) infection is a global public health issue and a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). HBV accounts for up to 42% of patients with cirrhosis and up to 56% of patients with HCC (Perz et al. 2006) causing ~700,000 deaths per year. As a result, HBV infection was ranked fifteenth among all causes of human mortality (Lozano et al. 2012).

Despite effective vaccines, studying HBV is crucial due to its global impact and the serious complications caused by CHB. In particular, HBV can persist in the liver through covalently closed circular DNA (cccDNA), which makes it difficult to cure and highlights the need for continued research to develop better prevention and curative strategies.

2.1.1-Epidemiology and transmission of HBV

Approximately 2 billion people have serological evidence of current or past infection with HBV and >350 million are chronically infected, although rates of HBsAg carriage vary greatly among different geographical areas. Indeed, the epidemiology of hepatitis B can be described based on the prevalence of hepatitis B surface antigen (HBsAg) in a population, broadly classified into high (8%), intermediate (2% – 7%) and low prevalence (2%) areas (*Figure 1*) (Papastergiou et al. 2015).

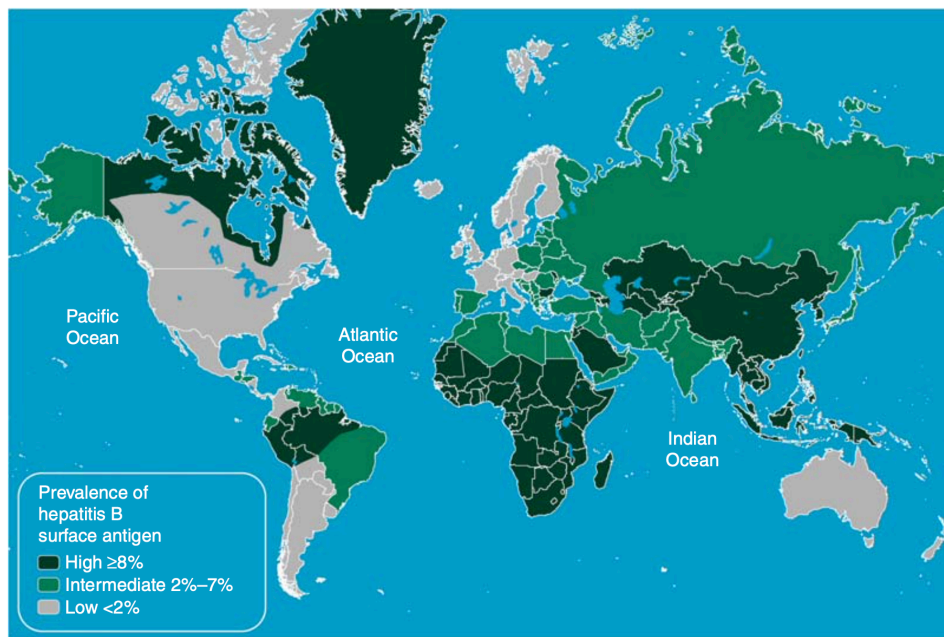


Figure 1: Global prevalence of hepatitis B virus infection (MacLachlan and Cowie 2015)

In countries where the prevalence of chronic HBV infection exceeds 8%, most affected individuals were infected perinatally or during early childhood, a period associated with a high risk of progression to chronic disease (Lavanchy 2004). It is estimated that 45%–60% of the global population lives in areas of high endemicity (Chemin and Pujol 2022). Areas where HBV is highly endemic include parts of Asia, sub-Saharan Africa, parts of the Middle East, the central Asian Republics, the Indian subcontinent, and some countries in Central and Eastern Europe.

Regions with intermediate HBV prevalence (ranging from 2% to 7%) include North Africa, the Middle East, parts of Eastern and Southern Europe, South Asia, and certain areas of Latin America. In these regions, transmission occurs both perinatally and horizontally. However, perinatal transmission is believed to be less common in countries with intermediate HBV prevalence, primarily because women of childbearing age in these regions are less likely to have highly infectious forms of the virus (Mahoney 1999).

Low-prevalence countries include regions such as North America, Western Europe, Japan, and Australia. In these areas, HBV transmission during childhood is rare, with most new infections occurring in adolescence or adulthood through sexual contact, injection drug use, or other blood-related exposures. Furthermore, migration from high- to low-prevalence regions significantly contributes to the burden of chronic HBV in low-prevalence countries, where most infected individuals are immigrants from endemic areas (Kowdley et al. 2012).

Vaccination, particularly in high-prevalence countries, has evidence to be highly effective in reducing HBV transmission. For example, in China, infant immunization led to a significant drop in HBsAg prevalence among children under 5, from 9.7% to 1.0%, thereby preventing millions of chronic infections. These results highlight the role of vaccination programs in changing HBV epidemiology (MacLachlan and Cowie 2015).

HBV can be detected in serum, urine, saliva, nasopharyngeal secretions, urine, tears, vaginal secretions, menstrual blood, and semen (Latthaphasavang et al. 2019). Therefore, HBV can be transmitted by perinatal transmission, horizontal transmission between children in infancy, percutaneous transmission, sexual exposure, or close person-to-person contact in the presence of open cuts (Nguyen et al. 2020). Perinatal transmission is the main route of HBV infection globally. Indeed, the risk of developing chronic infection is highest when the virus is acquired at birth, as newborns are more likely to develop immune tolerance to the virus (Yuen et al. 2018). The probability of developing CHB is inversely related to the age at which the infection is acquired. In fact, 80–90% of infants infected before the age of 1 develop chronic hepatitis B, while the risk decreases to 25–30% for those infected between ages 1 and 6. In contrast, immunocompetent adults have a 95% chance of clearing the virus and developing lifelong immunity upon re-exposure (Patel et al. 2024).

The development of chronic hepatitis B (CHB) is a complex process influenced by multiple factors that can promote disease progression. Viral factors include high levels of HBV replication, coinfection with hepatitis C, hepatitis D, or HIV, and the specific HBV genotype. Host-related factors include age at infection, duration of infection, male sex, presence of cirrhosis, use of tobacco or alcohol, and ethnic background, particularly among individuals of African or Asian (Trépo et al. 2014) (Fattovich et al. 2008).

HBV genetic variability is a key determinant in the progression and clinical outcome of CHB. In particular, the viral genotype has important prognostic value, as it can influence both the natural course of the disease and the patient's response to antiviral therapy. For example, genotypes A and B have been associated with a better response to interferon therapy compared to genotypes C and D. Additionally, certain genotypes, such as A, C, and D, appear to have a higher risk of progression to cirrhosis and HCC than others (McMahon 2009). This variability is largely driven by the lack of a proofreading mechanism in the HBV

reverse transcriptase, which introduces frequent mutations during viral genome replication and leads to the emergence of distinct genotypes and variants. (Z. Liu et al. 2021). In particular, HBV genotypes and sub-genotypes are classified based on differences in their DNA sequences. Genotypes differ from each other by more than 8%, while sub-genotypes show a difference of 4% to 8% (C.-L. Lin and Kao 2017). Ten different HBV genotypes (A-I) have been found, each with distinct geographical and ethnic distribution: Genotype A is mainly encountered in Africa, Northwestern Europe, and North America; Genotypes B and C are more prevalent in Asia; Genotype D is endemic in the Mediterranean basin and Eastern Europe, although it has a worldwide distribution; Genotype E is found in Western Africa; Genotype F is prevalent in South and Central America; genotype G has been reported in North and Central America and France; Genotype H in Central America and, genotype I has been detected in China, Vietnam, and Laos (Papastergiou et al. 2015).

2.1.2-HBV biology and genome organization

HBV virus belongs to the family of *Hepadnaviridae*, which is characterized by small enveloped viruses with icosahedral nucleocapsid that encloses a partially double-stranded DNA genome of 3.0–3.4 kb. All family members are hepatotropic, and the replication involves reverse transcription within nucleocapsids in the cytoplasm (Magnius et al. 2020). HBV was first discovered in an Australian aborigine by the detection of its antigen, currently known as surface antigen and originally called “Australia antigen” as reported by Blumberg in 1965 (Blumberg 1965).

The virus particles were visualized for the first time by an electron microscope in 1970 by Dane and colleagues. HBV particles are observed in three different types inside the serum of patients: spherical structures of 42 nm in diameter, those with a diameter of 22 nm, and filament structures of variable length that are 22nm in diameter.

The 42 nm particles, also called Dane particles, are the infectious virions consisting of a lipid membrane with three viral surface antigens (HBs): large (L-HBs), middle (M-HBs), and small (S-HBs), that surround a nucleocapsid composed of hepatitis B core protein (HBc), viral polymerase (Pol), and viral genome DNA (Tsukuda and Watashi 2020) (*Figure 2*).

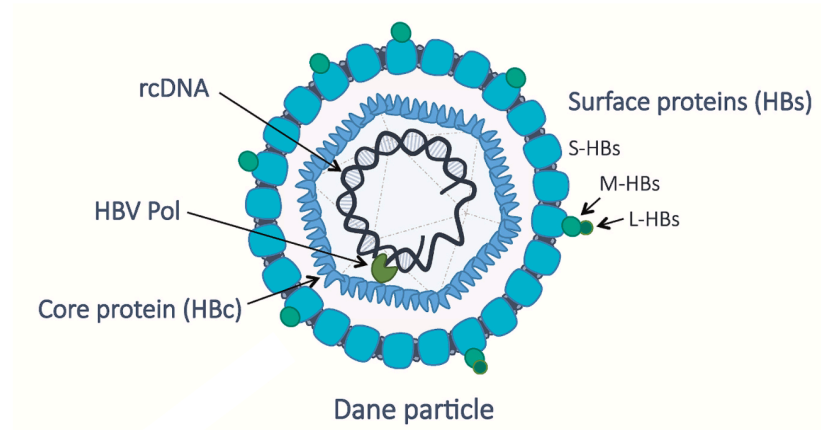


Figure 2: Schematic representation of infectious HBV particles (Dane particle) (Tsukuda and Watashi 2020)

The 22 nm particles, which are the most abundant in the serum of patients, consist of subviral particles (SVPs) that lack the nucleocapsid and are therefore non-infectious. In addition to these, other non-infectious particles are also produced during infection, including enveloped particles without viral genomes, particles containing viral RNA, and those lacking an envelope (Hu and Liu 2017).

The HBV genome DNA is a relaxed-circular DNA (rcDNA) of approximately 3.2 kb in length with a complete minus strand and an incomplete plus strand. The genome encodes four overlapping open reading frames (ORFs), C, P, S, and X, from which functional viral proteins are produced (Tsukuda and Watashi 2020).

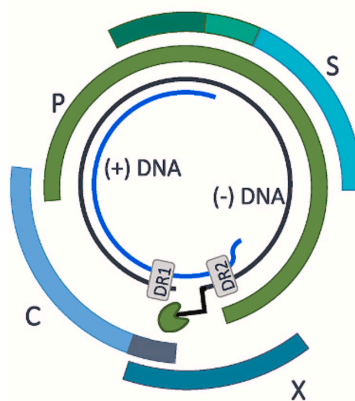


Figure 3: HBV genomic rcDNA and the encoded overlapping ORFs (C, P, S, X) (Tsukuda and Watashi 2020)

The largest open reading frame (ORF), known as ORF P, encodes the viral polymerase, which possesses reverse transcriptase (RT) activity. The second largest ORF, which is the S one, encodes the three viral envelope proteins: L-HBs, M-HBs, and S-HBs. ORF C encodes precore, also referred to as HBV and antigen (HBeAg), and the core protein, which compose the viral capsid. Finally, the ORF X encodes the HBV X protein (HBx), a small regulatory protein that has been shown to be required for HBV replication (Lamontagne et al. 2016).

After viral entry, the rcDNA is transported into the nucleus and converted into covalently closed circular DNA (cccDNA) by the host cell's DNA repair machinery. In HBV, viral persistence is thus maintained through the formation of cccDNA, which remains in the nucleus of infected hepatocytes as a stable episomal form (Beck and Nassal, s.d.). This cccDNA is not integrated into the host genome but instead forms a viral minichromosome by associating with histones H3 and H4. The transcriptional activity of cccDNA is tightly regulated by epigenetic modifications: acetylation of histones H3 and H4 promotes active transcription, while methylation, particularly of histone H4, represses it. Crucially, cccDNA serves as the template for all viral RNAs (W. Zhang et al. 2017a).

The cccDNA produces several capped and polyadenylated RNA transcripts, which are divided into genomic and subgenomic (*Figure 4*).

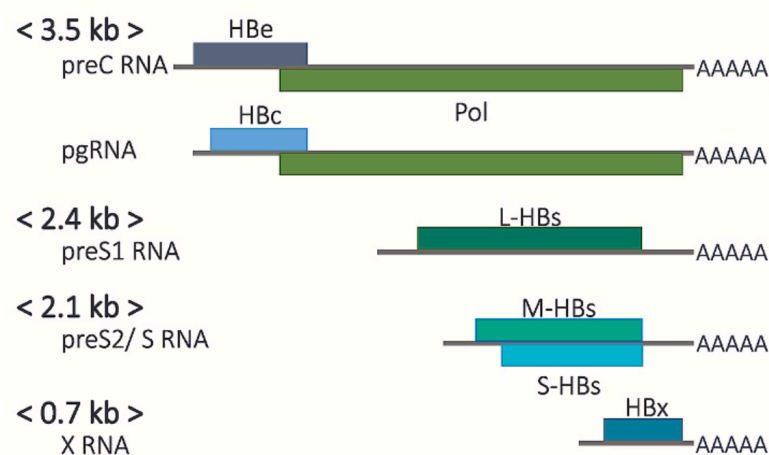


Figure 4: HBV RNAs produced by transcription of the cccDNA (Tsukuda and Watashi 2020)

Subgenomic RNAs serve only as templates for viral protein synthesis: the 0.7 kb transcript encodes HBx, while the 2.1 kb and 2.4 kb transcripts encode S-HBs, middle M-HBs, and large L-HBs. The 3.5 kb genomic RNAs act as mRNAs for the precore, core, and polymerase proteins. Among them, the pregenomic RNA (pgRNA) is multifunctional: it encodes both core and polymerase and also serves as the template for reverse transcription to form the viral DNA genome. Since the pgRNA is 3.5 kb, slightly longer than the 3.2 kb DNA genome, it contains terminal repeats. All HBV transcripts share a common polyadenylation site; as a result, the HBx sequence is found at the 3' end of all HBV mRNAs, while only the longest transcript contains unique upstream sequences (Seeger and Mason 2015).

All these genomic and subgenomic HBV RNAs serve as templates for the production of viral proteins. These include: the precore protein that is the precursor of and antigen (HBeAg), the core protein (HBcAg), the viral polymerase, the X protein (HBx), and the three envelope proteins known as HBsAg. Together, these proteins are essential for viral replication, assembly, and interaction with the host immune system.

2.1.2.1-HBV Precore Protein

The precore protein is the precursor of hepatitis B and antigen (HBeAg), a secreted protein that circulates in the blood of infected individuals, separate from the viral particles (Gerlich et al. 2020). It is initially produced as a 25-kDa precursor (p25) with a 29-amino acid signal at its beginning, which helps direct the protein into the endoplasmic reticulum (ER). Inside the ER, the first 19 amino acids are removed by a cellular enzyme, resulting in a smaller 22-kDa intermediate form (p22) (Tsai and Ou 2021). After p22 is formed, it is redirected to the Golgi apparatus, where its C-terminal arginine-rich domain (ARD) functions as a cleavage site for a furin-like protease. This final cut results in the mature and secreted form of HBeAg, which is about 17 kDa in size (Messageot et al. 2003) (*Figure 5*).

Therefore, HBeAg is secreted by infected cells but also exists in multiple intracellular forms during its processing. These intermediates, found in compartments like the ER and Golgi, suggest that HBeAg may have additional roles inside the cell.

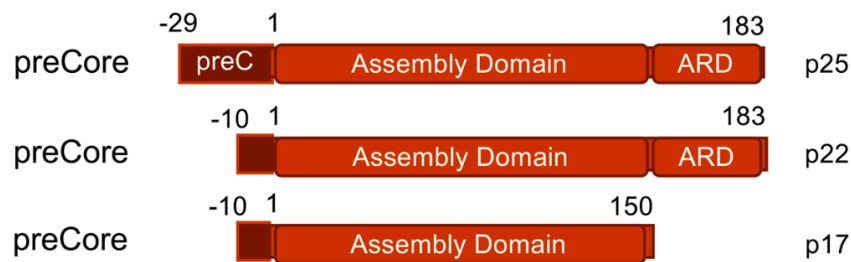


Figure 5: Schematic representation of the HBV precore translational products (Prange 2022)

The exact functions of the precore protein and HBeAg are still not fully understood, but neither is essential for viral replication (Tong et al. 1991). Instead, HBeAg is believed to play a role in modulating the host immune response, helping the virus to persist in the body. Unlike other viral components, HBeAg is not part of the infectious HBV particle, and its secretion may serve to distract the immune system, reducing its ability to target the actual virus (Kramvis et al. 2018). Furthermore, HBeAg is an important marker of active viral replication, as its presence in the blood generally correlates with high levels of HBV DNA. Seroconversion from HBeAg to anti-HBe is often seen as a sign of reduced viral replication and is considered a key step in the transition to the inactive carrier state, with normal liver enzymes and minimal liver inflammation (Liaw 2009).

2.1.2.2-HBV Core Protein

The 21 kDa hepatitis B core protein (HBcAg) is used as the structural backbone of the HBV capsid. It is translated from the pgRNA and is composed of two main regions: the N-terminal assembly domain (first 149 amino acids) (Birnbaum and Nassal 1990), and the C-terminal domain (CTD), an arginine-rich region of 34–36 amino acids. This CTD is heavily phosphorylated, and its phosphorylation–dephosphorylation regulates several stages of the HBV life cycle, including reverse transcription, genomic replication, cccDNA stability, and intracellular trafficking (Melegari et al. 2005). Therefore, while the primary and best-characterized function of the core protein is nucleocapsid assembly, recent studies have highlighted several additional roles that can influence HBV life cycle.

The core protein is not static in its localization, indeed, it moves between the nucleus and cytoplasm of infected cells. This dynamic trafficking is clinically relevant: in patients with mild or asymptomatic disease, core accumulates in the nucleus, whereas in chronic active hepatitis, it is predominantly found in the cytoplasm. This compartmentalization pattern may

reflect the stage or severity of infection and could have diagnostic or prognostic significance (Chu et al. 1995).

2.1.2.3 - Polymerase

The hepatitis B virus (HBV) polymerase protein (Pol) is a 90 kDa enzyme. It consists of three functional domains and a variable spacer region. The N-terminal portion harbors the terminal protein (TP) domain, which plays a crucial role in several aspects of viral genome replication initiation. Indeed, this domain is responsible for: binding to the pregenomic RNA (pgRNA), RNA encapsidation, and protein-priming processes (Weber et al. 1994). P domain is followed by a variable spacer region that separates it from the RT domain. Notably, mutagenesis studies have demonstrated that the vast majority of amino acids within this spacer region can be altered without compromising polymerase function (Radziwill et al. 1990). However, three conserved cysteine residues located at the C-terminal end of the spacer, along with a fourth cysteine at the N-terminal boundary of the RT domain, appear to be essential for the enzymatic activity of the polymerase (S. Kim et al. 2009).

The reverse transcriptase (RT) domain of the HBV polymerase is responsible for viral genome replication, primarily through the reverse transcription of the pregenomic RNA (pgRNA) to synthesize the negative (-)-strand of the DNA genome, followed by the use of this strand as a template for the synthesis of the positive (+)-strand. This domain exhibits considerable sequence and structural homology to the RT enzymes of other retroviruses (Xiong and Eickbush 1990).

The final domain of the polymerase is the ribonuclease H (RH) domain, which facilitates the degradation of the pgRNA template during the synthesis of the (-)-strand DNA (Tavis et al. 2013).

2.1.2.4 - X protein

HBx is the only regulatory protein encoded by HBV and consists of a 154aa, 17 kD protein, that is encoded by the smallest HBV ORF. Various studies have provided considerable evidence that HBx plays an essential role during HBV replication. In particular, studies have demonstrated that HBx associates with covalently closed circular DNA (cccDNA) (Belloni et al. 2009), is essential for transcriptional activation from the cccDNA template (Seeger and Sohn 2014), and that HBx-mediated downstream effects are necessary to sustain efficient

viral replication. Structurally, the HBx protein consists of two main regions: an N-terminal domain that acts as a negative regulator, and a C-terminal domain that functions in transactivation or coactivation. The C-terminal domain modulates host cell signaling pathways in ways that support HBV replication. The most well-characterized cellular binding partner of HBx is the damage-specific DNA binding protein 1 (DDB1). The interaction between HBx and DDB1 is essential for HBV replication, as it enables the targeting and degradation of the SMC5/6 complex. This latter is a restriction factor for HBV because it prevents HBV transcription from cccDNA (Li et al. 2010). Furthermore, HBx can modulate calcium, apoptosis, and activate transcription factors, including activator proteins, nuclear factor of activated T cells (NFAT) (Lara-Pezzi et al. 1998), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Um et al. 2007).

2.1.2.5 - HBV Surface antigens

HBV encodes three envelope proteins, or surface antigens, that compose the viral envelope: large (L), middle (M), and small (S) surface antigen. The smallest envelope protein, or S, is 226 amino acids (aa) in length and shares the C-terminal region of the two longer envelope proteins. The M protein contains the S sequence with a 55aa N-terminal extension known as preS2. The L protein is the largest of the envelope proteins, contains S, preS2, and an additional 108aa or 119aa (depending on the genotype) N-terminal extension known as preS1 that is the receptor-binding region (Lamontagne et al. 2016) (*Figure 6*).

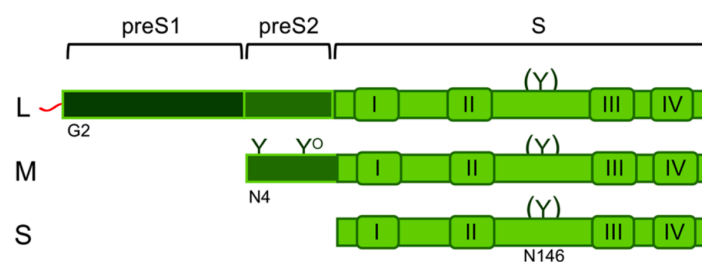


Figure 6: Schematic representation of the HBV envelope ORF (Prange 2022)

Although the three HBV envelope proteins are structurally related, they have distinct functional roles. The S protein constitutes the major structural component of the viral envelope, forming the scaffold essential for particle assembly. The L protein, in contrast, is critical for both viral infectivity and the envelopment of nucleocapsids. However, the M protein appears to have no essential function, as its deletion does not appear to impair virion

production or infectivity (Le Seyec et al. 1998). In particular, the L protein can have two conformations that correspond to its two functions, which differ in the orientation of the N-terminal domain. In the first conformation, essential for capsid binding and assembly of mature virions, the preS1 and preS2 domains face the cytosol. In the second conformation, required for attachment and entry of HBV, the N-terminal domain is translocated into the lumen of the ER, resulting in its exposure on the surface of viral particles (Taylor 2013). In addition to their role in the formation of infectious virions, the surface antigens are key component for the production of non-infectious subviral particles (SVPs). These SVPs are produced in large quantities by HBV-infected cells and are composed primarily of S-HBsAg, with variable amounts of M-HBsAg and minimal L-HBsAg. The concentration of SVPs in the serum of infected individuals can exceed that of infectious virions by up to 10,000-fold (Ganem 2004). Although the exact function of SVPs is not fully understood, they are thought to help HBV evade the immune response by acting as antibody decoys and promoting immune tolerance, thereby supporting chronic infection (Bruns et al. 1998).

2.1.3 - HBV Viral life cycle

2.1.3.1 - Entry

The HBV life cycle (*Figure 7*) begins with HBV entry into host hepatocytes, a multistep process that involves both low-affinity and high-affinity interactions with host cell surface molecules. The initial attachment of HBV to hepatocytes is mediated by non-specific, low-affinity binding to heparan sulfate proteoglycans (HSPGs), such as glypican 5 (Verrier et al. 2016). This initial interaction facilitates the subsequent engagement of the virus with its high-affinity receptor, which was identified as the human sodium taurocholate cotransporting peptide (hNTCP). NTCP is a liver-specific bile acid transporter that is exclusively expressed on the basolateral membrane of hepatocytes and acts as the functional receptor for both HBV and hepatitis D virus (HDV) (Yan et al. 2012). The recognition of NTCP as a receptor was based on its ability to bind specifically to amino acids 2–48 of the HBV preS1 domain contained in L-HBs (Ni et al. 2014). In addition, N-myristoylation of the preS1 domain has been shown to be essential for viral infectivity. Indeed, a synthetic myristoylated peptide comprising the first 47 amino acids of the preS1 domain can bind to hNTCP and effectively block HBV binding, serving as a potential antiviral agent (Gripon et al. 2004).

Following receptor engagement, HBV is internalized into hepatocytes through an endocytosis-dependent mechanism (Yamada et al. 2012). Although the precise mechanisms of viral internalization and subsequent membrane fusion remain poorly understood, it is believed that fusion occurs between the viral envelope and vesicular membranes derived from the host cell. Further studies have revealed that NTCP undergoes oligomerization and that the oligomeric state of NTCP modulates its ability to mediate HBV internalization (Fukano et al. 2019). These findings suggest that not only the presence, but also the conformational state of NTCP, is critical for productive infection.

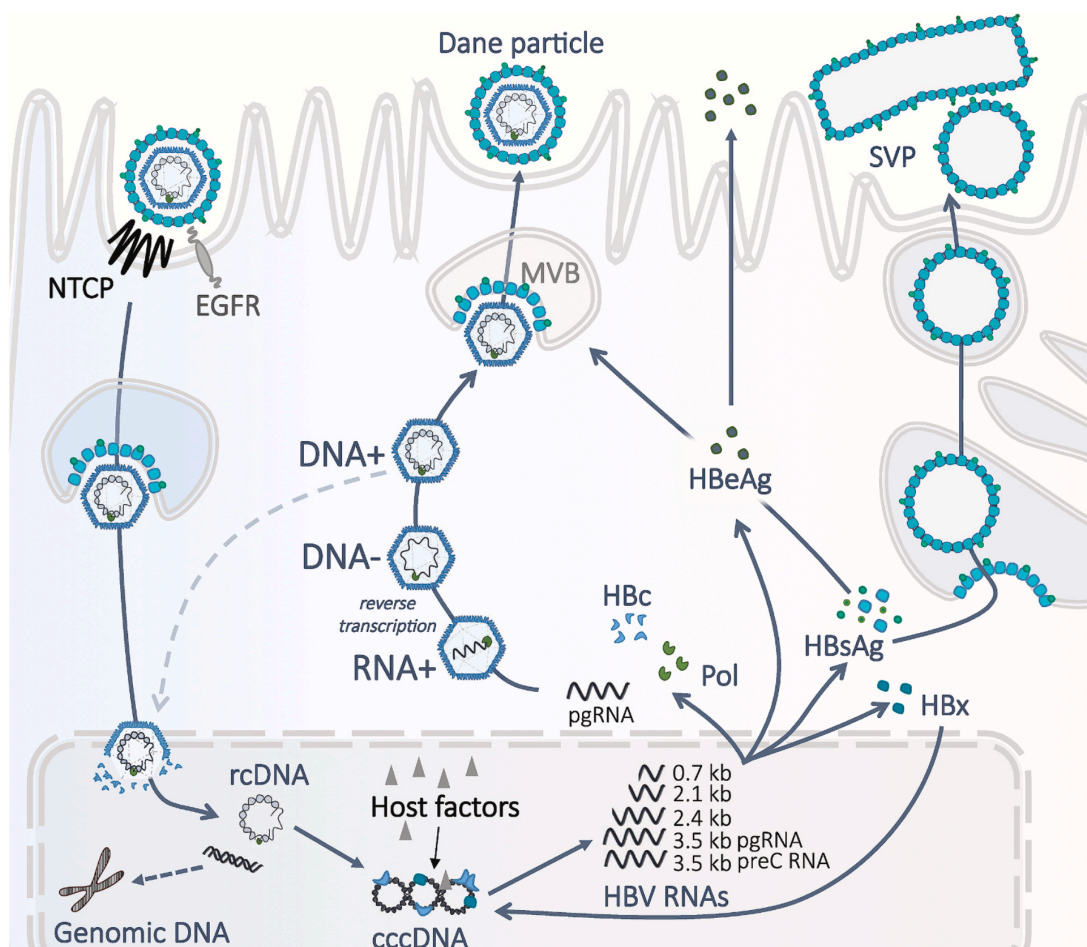


Figure 7: Schematic representation of HBV life cycle (Tsukuda and Watashi 2020)

2.1.3.2 - Nuclear import and conversion to cccDNA

Once the HBV entry occurs, the incoming nucleocapsid in the cytoplasm is directed to the nucleus along with the microtubules (Rabe et al. 2006) and imported into the nucleus through the nuclear pore complex in an importin-dependent manner (Rabe et al. 2003).

Following nuclear import, the rcDNA of HBV is converted into covalently closed circular DNA (cccDNA), a critical step in the viral life cycle. The single-stranded regions present in the rcDNA are modified either by the activity of the viral polymerase or via host cellular repair mechanisms. This process results in gaps in both DNA strands, which are filled and ligated, leading to the formation of cccDNA (G.-H. Wang and Seeger 1992). In particular, the enzymes involved in filling the gaps in rcDNA are DNA polymerase κ and α (Pol κ and Pol α), DNA ligase 1 and 3 (LIG1 and LIG3), and topoisomerase I and II (TOP1 and TOP2) (Sheraz et al. 2019) (L. Tang et al. 2019).

Once formed, cccDNA is assembled into a minichromosome structure within the nucleus that involves host cellular histones, where it is organized into a nucleosome-bound episome. The interactions with histones are thought to influence both the structural conformation of the cccDNA and its epigenetic regulation, thereby affecting viral transcription and persistence (Pollicino et al. 2006). Post-translational modifications of histones associated with cccDNA play a crucial role in regulating HBV transcription. Specifically, histone 3 (H3) shows high levels of activating marks, such as trimethylation at lysine 4 (H3K4me3) and acetylation at lysines 27 and 122 (H3K27ac and H3K122ac), which are enriched at specific regions of the HBV genome. These modifications are typically associated with active transcription. In contrast, repressive histone marks such as H3K27me3 and H3K9me2 are present at very low levels, even at transcriptionally silent HBV promoters (Tropberger et al. 2015). There are also accumulating reports of histone modification enzymes and other factors directly or indirectly recruited onto the cccDNA minichromosome to regulate viral transcription. Such histone modification enzymes include: histone acetyltransferases, histone deacetylases, histone methyltransferases, and demethylases (Yang et al. 2019) (W. Zhang et al. 2017b). Importantly, cccDNA-associated histone modifications have been shown to be directly targeted by the antiviral effects of interferon (IFN). Specifically, IFN- α induces hypoacetylation of histones bound to cccDNA and promotes the recruitment of transcriptional repressors, leading to transcriptional silencing of the viral genome (Belloni et al. 2012).

In addition to cccDNA formation, a part of the HBV DNA is integrated into the host genome. A cell culture study showed that the integration occurs as fast as within a week after infection

(Tu et al. 2018). The integrated HBV DNA is replication-incompetent but can act as a template for the production of HBs, which is thought to be related to HBV-specific immune tolerance and the development of HBV-related pathogenesis (Tsukuda and Watashi 2020).

2.1.3.3 - Transcription and Translation

The subgenomic RNAs, along with the pgRNA and precore RNA, are transcribed by the host cellular RNA polymerase II using cccDNA as the template. All transcripts possess a 5' cap structure and share a common 3'-terminal polyadenylation site, and each functions as messenger RNA (mRNA) for the synthesis of viral proteins (Beck and Nassal, 2007).

In addition to the previously described epigenetic regulation, transcription from HBV cccDNA is also governed by the recruitment of host transcription factors to viral promoter and enhancer regions. These regions contain binding sites for numerous factors, including the liver-enriched nuclear receptors hepatocyte nuclear factor 3 and 4 (HNF3, HNF4), retinoid X receptor alpha (RXR α), peroxisome proliferator-activated receptor alpha (PPAR α), and farnesoid X receptor (FXR) (Tsukuda and Watashi 2020). In addition to transcription factor recruitment, HBV transcription is strongly influenced by HBx epigenetic regulation of cccDNA. In the absence of HBx, cccDNA becomes transcriptionally silenced, characterized by reduced H3 acetylation and H3K4me3, increased H3K9me2/3, recruitment of heterochromatin protein 1 (HP1), and subsequent chromatin condensation. HBx expression reverses this silencing by restoring H3K4me3 levels and preventing HP1 recruitment, maintaining a transcriptionally active chromatin state (Rivière et al. 2015).

Following transcription, HBV mRNAs are exported from the nucleus to the cytoplasm via the host cell's canonical mRNA export pathways. Once in the cytoplasm, these transcripts are translated by the host ribosomal machinery (Lamontagne et al. 2016).

2.1.3.4 - Reverse transcription and viral replication

For efficient HBV replication, the stability and secondary structures of the pgRNA are critical determinants. In particular, the pgRNA contains a conserved stem-loop structure, termed epsilon (ϵ), located at both its 5' and 3' ends (*Figure 8*). This structural element is essential for encapsidation of pgRNA into nucleocapsids and has also been implicated in modulating RNA stability. Recent studies have demonstrated that the zinc finger antiviral protein (ZAP) recognizes HBV RNAs through a region encompassing the epsilon element,

promoting their degradation primarily within the nucleus. This antiviral effect is further enhanced by IFN treatment (Mao et al. 2013).

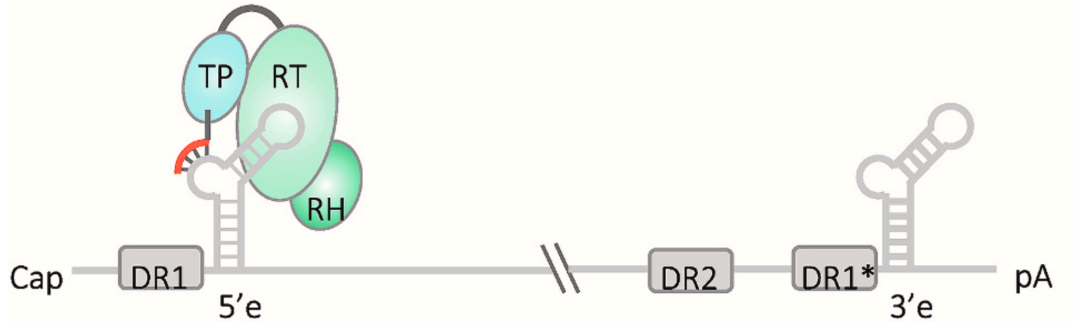


Figure 8: pgRNA organization. The pgRNA is shown with some major cis-elements: ϵ , direct repeat 1 and 2 (DR1, DR2) (Tsukuda and Watashi 2020)

RNA encapsidation in HBV requires the action of HBc, Pol, and the pgRNA. Newly synthesized HBc monomers first assemble into dimers, and subsequently, 90 or 120 of these dimers self-assemble to form an icosahedral capsid (Böttcher et al. 1997). During encapsidation, the TP domain of Pol specifically recognizes and binds the ϵ stem-loop structure located at the 5' terminus of pgRNA, forming a ribonucleoprotein complex that is then incorporated into the assembling capsid (Pollack and Ganem 1994). This process is facilitated by host molecular chaperones, including heat shock protein 90 (Hsp90), Hsp40, and heat shock cognate 70 (Hsc70), which interact with Pol to promote its conformational maturation (Beck and Nassal 2003) (Stahl et al. 2007). Additional host factor reported to be determinant for encapsidation is eukaryotic translation initiation factor 4E (eIF4E) (S. Kim et al. 2010). Following RNA encapsidation, synthesis of the viral genome proceeds through several steps within the nucleocapsid (Figure 9).

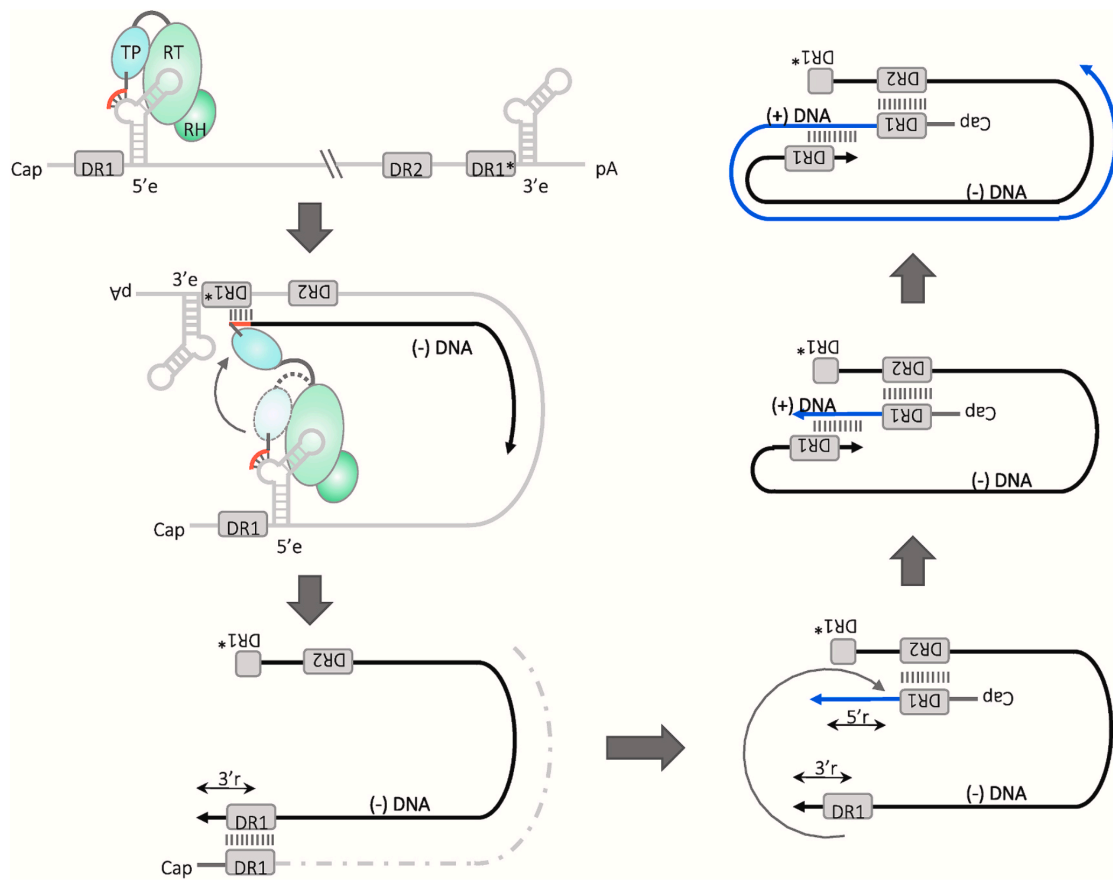


Figure 9: Schematic representation of reverse transcription, RNA degradation, and DNA synthesis within HBV nucleocapsids. The viral polymerase (Pol), comprising the terminal protein (TP), reverse transcriptase (RT), and RNase H (RH) domains, is recruited to the 5'-terminal epsilon (ε) element, where it initiates the replication (Tsukuda and Watashi 2020)

The epsilon element in pgRNA contains an internal bulge with the sequence 5'-UUC-3', which serves as the template for priming. In the TP domain of Pol, a tyrosine residue at position 63 acts as the protein primer (X. Wang and Hu 2002) (Figure 10).

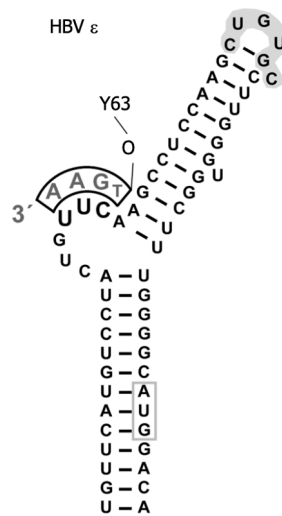


Figure 10: Secondary structures of HBV ϵ signal (Beck and Nassal, 2007)

The first dGTP is covalently attached to the hydroxyl group of Y63 to initiate priming, followed by the addition of two dAMP residues to generate the sequence 5'-dGAA-3'. The produced Pol-dGAA complex is then translocated to the complementary direct repeat 1 (DR1) sequence at the 3' end of pgRNA, allowing the synthesis of minus-strand DNA (L. Lin et al. 2008).

The minus-strand DNA is extended to the 3' end of the pgRNA, resulting in a full-length minus-strand DNA that contains an additional terminal redundancy. Simultaneously, the pgRNA template is degraded by the RNase H domain, leaving a short RNA fragment that includes the capped 5' terminal region and the 5' direct repeat 1 (DR1) sequence. This RNA fragment is subsequently translocated to the direct repeat 2 (DR2) sequence at the 3' terminus, where it serves as a primer for extension to the 5' end of the minus-strand DNA. Due to the presence of terminal redundancy, the elongation of the 3' end of the plus-strand DNA can switch to the 3' redundant sequence on the minus-strand DNA, facilitating further extension of the plus-strand DNA and ultimately resulting in the formation of relaxed circular DNA (rcDNA) (Tsukuda and Watashi 2020).

The replication takes place within core particles and culminates in the synthesis of encapsidated, partially double-stranded relaxed circular DNA (rcDNA) with the viral polymerase covalently attached to the 5' end of the negative minus-strand DNA. Following DNA synthesis, the nucleocapsid can follow two pathways: in the first, it is transported back

to the nucleus, where it contributes to the amplification and maintenance of a stable pool of covalently closed circular DNA (cccDNA); in the second the nucleocapsids are enveloped and, mature virions are secreted from the infected cells (Pourcel and Summers, 1986) (Figure 11).

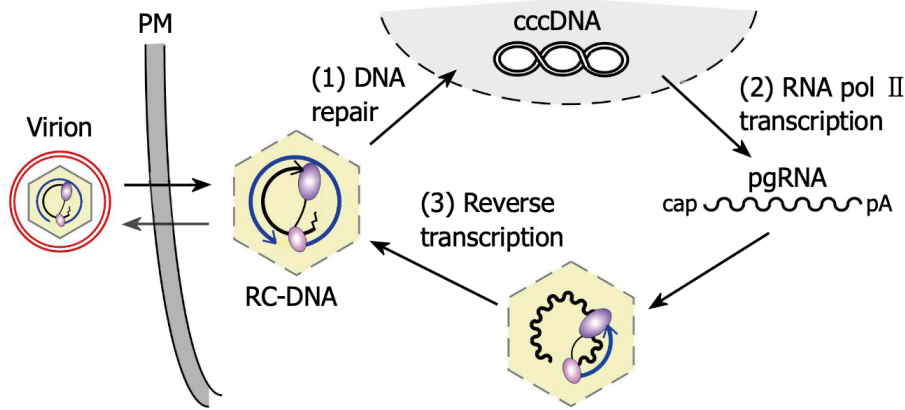


Figure 11: Replication cycle of the HBV genome. New replication cycles lead to intracellular cccDNA replenishment; alternatively, the rc-DNA containing nucleocapsids are enveloped and released as virions (Beck and Nassal, 2007)

The first pathway involving cccDNA recycling is influenced by the levels of envelope proteins: reduced amounts of HBsAg promote the shuttling of the nucleocapsid to the nucleus. In particular, L-HBsAg levels directly affect the transport back to the nucleus (Lentz and Loeb 2011). This process generates a pool of cccDNA, typically containing fewer than 10 copies per cell, which can persist for years. The estimated half-life of a single cccDNA molecule ranges from 33 to 57 days, highlighting its critical role in sustaining HBV persistence (Laras et al. 2006).

2.1.3.5 - Assembly and release

The second potential route of HBV nucleocapsids is envelopment by HBV envelope glycoproteins inserted in the endoplasmic reticulum (ER) membrane. The overall secretion rate of infectious HBV Dane particles has been estimated to be only 1–10 virions per cell per day (Nowak et al. 1996).

The release of Dane particles was initially believed to occur through the same secretory route as the highly abundant subviral particles (SVPs). In this model, envelope proteins located in the ER–Golgi intermediate compartment would bind DNA-containing capsids, enabling their entry into the lumen and subsequent secretion. More recent evidence, however,

indicates that mature HBV virions use a distinct route that depends on the endosomal sorting complex required for transport (ESCRT) machinery, which drives the formation of multivesicular bodies (MVBs) (Watanabe et al. 2007).

Interestingly, subviral particles of spherical morphology, predominantly composed of S-HBsAg, are released from the ER via the conventional secretory pathway (Patient et al. 2009), whereas filamentous SVPs that additionally incorporate L-HBsAg share the ESCRT-dependent egress route (Jiang et al. 2016). Collectively, these observations highlight the close interplay between HBV egress mechanisms and the host cell's membrane sorting machinery.

2.1.4 – Natural history and clinical manifestation

2.1.4.1 – Acute and chronic infection

It has been established that, upon entering the circulatory system, HBV possesses a remarkable ability to target and infect parenchymal liver cells. Once infection is initiated, HBV replication and intrahepatic spreading proceed rapidly, with an estimated viral doubling time of approximately 2–4 days (Whalley et al. 2001). Experimental infection models have further shown that, depending on the inoculum dose, HBV can spread to 100% of hepatocytes within 7–10 weeks, without any detectable signs of hepatocellular damage. Importantly, the eventual outcome of infection is not strictly determined by the inoculum dose. Indeed, even minimal viral exposure can result in chronic infection accompanied by immunopathology. Disease progression is governed by the interplay between the kinetics of viral spread and the timing of adaptive immune priming, particularly the activation of HBV-specific CD4⁺ T cells (Asabe et al. 2009).

The clearance of HBV-infected hepatocytes and the prevention of further intrahepatic spread require a combination of cytolytic and noncytolytic mechanisms, as well as coordinated actions of both the innate and adaptive immune responses (Xia and Protzer 2017) (*Figure 12*).

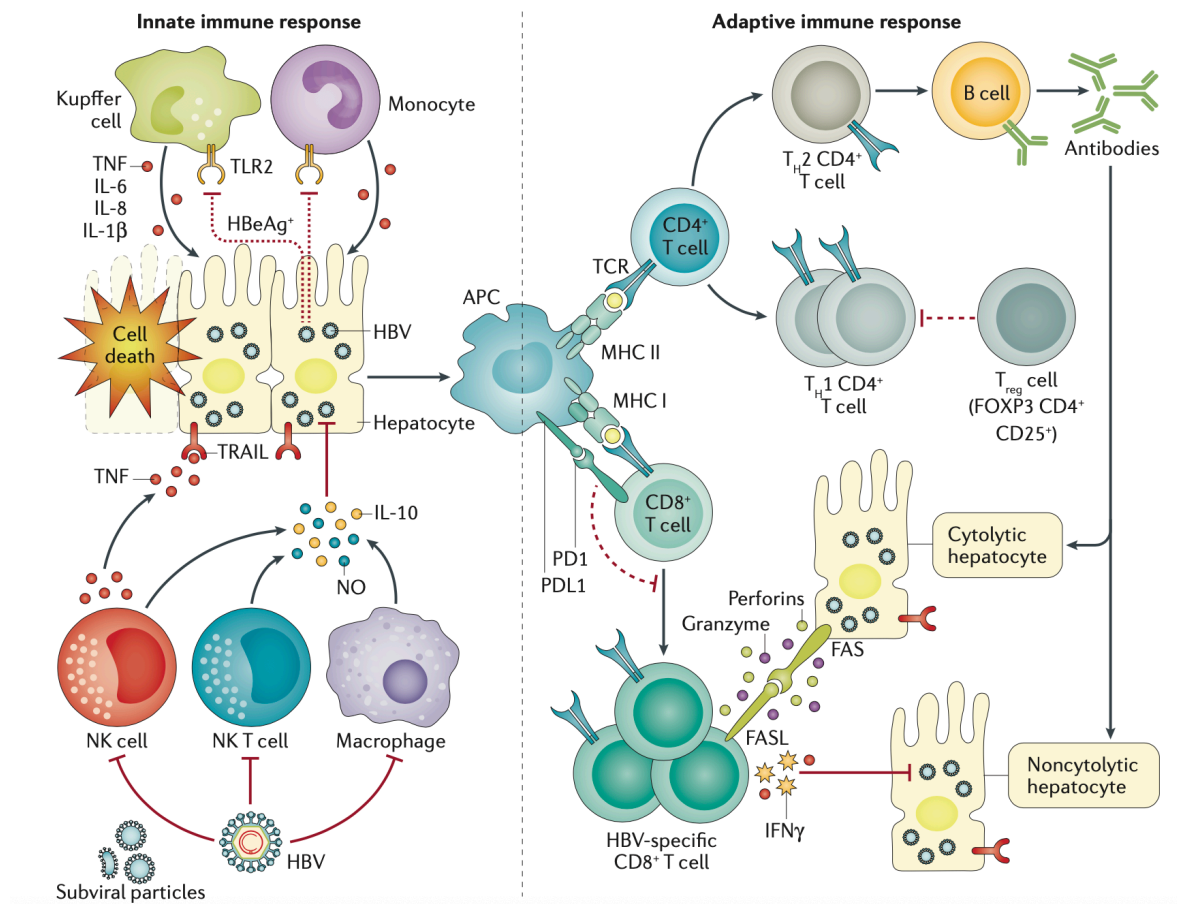


Figure 12: HBV-specific immune response involving both innate and adaptive immune systems (Yuen et al. 2018)

During infection, HBV pathogen-associated molecular patterns (PAMPs) are detected by innate immune cells, including Kupffer cells and monocytes, which respond by releasing antiviral cytokines such as tumour necrosis factor (TNF) and interferon- γ (IFN γ). In turn, HBV counteracts this early defense through the expression of its own proteins, most notably HBeAg and HBsAg. HBeAg suppresses Toll-like receptor 2 (TLR2) signalling in Kupffer cells, monocytes, and hepatocytes, thereby promoting viral persistence. Excessive production of HBsAg subviral particles further compromises innate immunity. This occurs by reducing the functional activity of natural killer (NK) cells, NK T cells, and macrophages, through the induction of the immunosuppressive cytokine interleukin-10 (IL-10) (Yuen et al. 2018). Furthermore, an excessive production of subviral particles enriched in HBsAg may function as a decoy for HBV-specific humoral immunity. This phenomenon has been implicated in the induction of virus-specific T cell anergy, defined as functional exhaustion

resulting from persistent stimulation of antigen-specific T cell receptor signalling pathways, and in the subsequent deletion of these cells (Wong et al. 2015) (Isogawa et al. 2013). The resolution of HBV infection and the progression of disease are closely linked to the activation of a robust T cell response targeting the entire viral proteome (Yuen et al. 2018). Indeed, virus-specific CD8⁺ T cells mediate clearance of infected cells, B cells secreting neutralizing antibodies against HBsAg (anti-HBs) that block further infection, and CD4⁺T cells supporting effective viral clearance (Guidotti et al. 1994). In contrast, chronic infection is characterized by a markedly weakened HBV-specific T cell response (Yuen et al. 2018). As is to be expected, persistent chronic HBV infection occurs predominantly when the virus is acquired at birth or during the first two years of life, a period in which the immune system is still immature and lacks the capacity to coordinate the response required for clearance of acute infection. As a result, the virus is more likely to establish long-term persistence, thus developing CHB.

2.1.4.2 – Diagnosis and biomarkers

Serological markers are essential for the diagnosis and differentiation of acute and chronic HBV infection. Among adults with acute HBV infection, only 5 to 10% progress to chronic infection, and only approximately one-third of these individuals develop clinical symptoms such as fever, fatigue, malaise, abdominal pain, or jaundice during the acute phase (L. S. Y. Tang et al. 2018). Several distinct biological markers are available for the evaluation and diagnosis of HBV infection, including HBsAg, HBeAg, antibodies to the HBsAg (anti-HBs), antibodies to the HBcAg (anti-HBc), antibodies to the HBeAg (anti-HBe), and HBV DNA.

The appearance of antibodies to HBV antigens follows characteristic patterns during acute hepatitis B (*Figure 13*). Within a few weeks after the appearance of viral markers, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels begin to rise, and jaundice may develop. HBeAg is typically cleared early, often at the peak of clinical illness, and is thus considered a marker of HBV replication and infectivity. In contrast, HBsAg and HBV DNA generally persist in the serum throughout the symptomatic phase and are eliminated upon recovery, and thus it is considered a marker of chronicity (Jake and Marc 2002). Antibody to the core antigen (anti-HBc) usually emerges shortly before the onset of clinical symptoms, initially in the immunoglobulin M (IgM) class. IgM anti-HBc

titers subsequently decline as immunoglobulin G (IgG) anti-HBc becomes predominant. Antibody to the envelope antigen (anti-HBe) typically appears shortly after HBeAg clearance, often coinciding with the peak of clinical illness. The loss of HBeAg, accompanied by the emergence of anti-HBe, is considered a favorable serological indicator of the onset of recovery. Antibody to the surface antigen (anti-HBs) arises later in the course of infection, usually during recovery or convalescence, following the clearance of HBsAg. Anti-HBs persists after recovery and is associated with protective immunity against HBV. Nonetheless, approximately 10 to 15 percent of patients who recover from hepatitis B fail to develop detectable anti-HBs, retaining anti-HBc as the only serological marker of previous infection. For this reason, testing for anti-HBc remains the most reliable method of identifying past HBV infection, whereas anti-HBs testing is primarily used to assess immunity and response to HBV vaccination (Tabor et al. 1981).

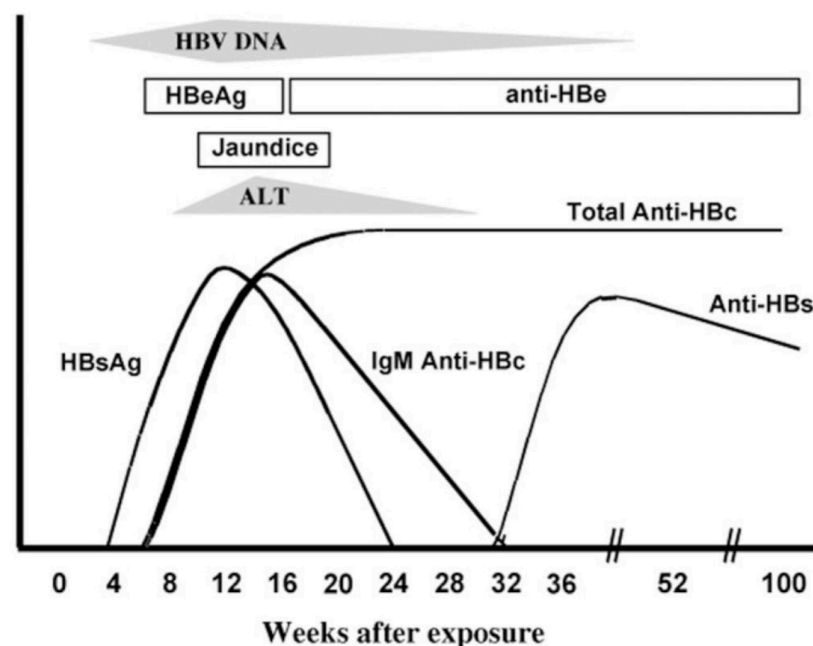


Figure 13: The clinical course and serological profile of acute hepatitis B (Liang 2009)

Patients who progress to chronic hepatitis B exhibit an initial serological profile similar to that observed in acute infection, characterized by the appearance of HBV DNA, HBsAg, HBeAg, and anti-HBc (*Figure 14*). In these individuals, however, viral replication persists, and HBsAg, HBeAg, and HBV DNA remain detectable in the serum, often at high titers. The clinical course of chronic hepatitis B is highly variable. Most patients remain HBsAg-

positive for many years, and often for life, and exhibit some degree of chronic liver injury that may progress to significant fibrosis and cirrhosis. Furthermore, individuals with chronic HBV infection are at a substantially increased risk of developing hepatocellular carcinoma (Liang 2009). The detection of HBsAg in the absence of IgM anti-HBc is suggestive of chronic hepatitis B; however, a definitive diagnosis typically requires demonstration of HBsAg persistence for a minimum of six months (Krugman et al. 1979). HBV DNA testing is a valuable tool for assessing the degree of viral replication, with important implications for prognosis and the decision to initiate antiviral therapy. Persistently elevated HBV DNA levels after the acute phase suggest inadequate viral control and progression to chronic infection, a condition often accompanied by sustained levels of ALT throughout the disease course (Liang 2009).

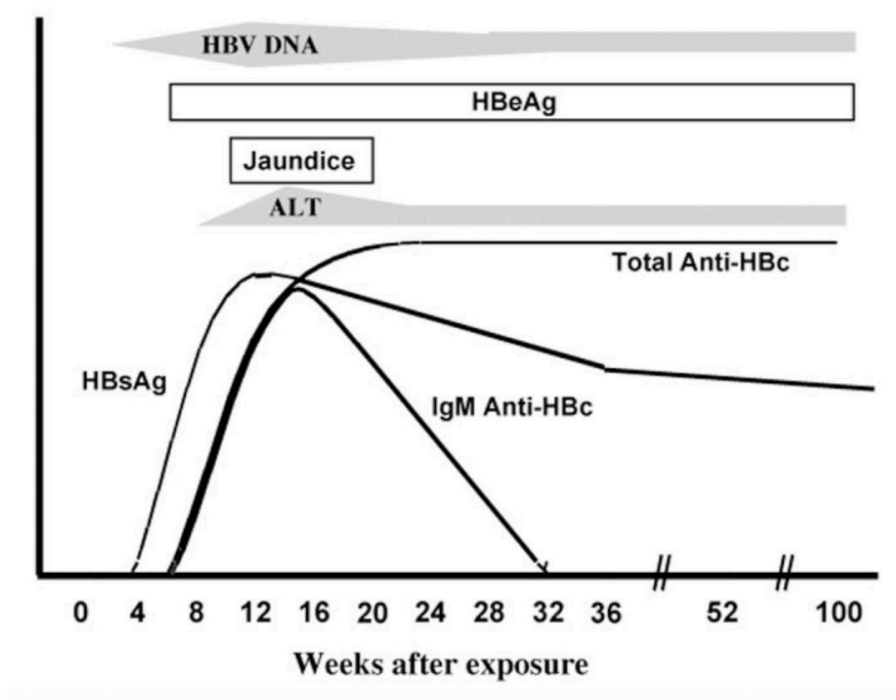


Figure 14: The clinical course and serological profile of chronic hepatitis B (Liang 2009)

2.1.4.3 – Phases of chronic infection

New terminology has been proposed for the phases of chronic HBV infection. The previously used terms “immune tolerance,” “immune active” or “clearance,” and “immune control” or “residual” phases have been replaced by “HBeAg-positive chronic infection,” “HBeAg-positive chronic hepatitis,” and “HBeAg-negative chronic infection,” respectively

(Lampertico et al. 2017). These distinct phases are defined by the interplay between HBV viral replication, as reflected by serum HBV DNA levels, and the activity of liver injury, as indicated by serum ALT concentrations, as well as by the presence of HBeAg and anti-HBe antibodies (*Figure 15*).

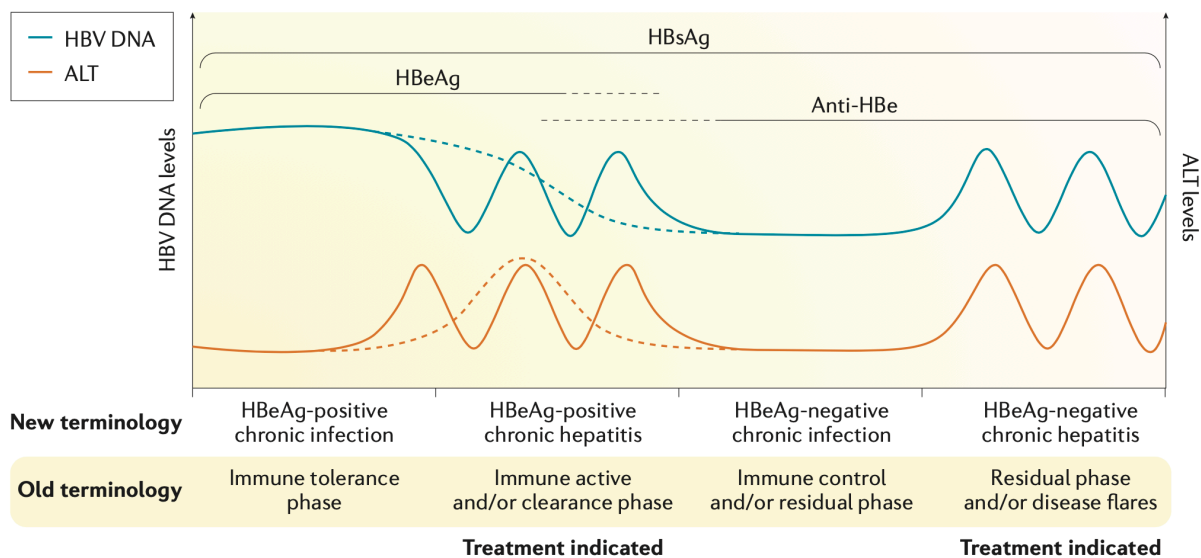


Figure 15: Chronic hepatitis B disease phases (Yuen et al. 2018)

The initial immune tolerance phase, also referred to as HBeAg-positive chronic infection, typically manifests during chronic infection acquired in childhood. This phase is characterized by markedly elevated viral loads, with HBV DNA levels frequently exceeding 10^7 international units (IU)/mL, near-normal liver histology resulting from minimal host immune-mediated activity, and persistent serum positivity for HBeAg (Yuen et al. 2018b). Studies of this disease phase have also demonstrated clonal expansion of hepatocytes and the presence of identifiable HBV DNA integrations, which may promote HCC by transactivating human oncogenes (Mason et al. 2016). Detectable HBV-specific T cell immunity may still be compatible with a state of immune tolerance, which underlies viral persistence and chronic infection. In this context, immune tolerance to HBV is operationally defined as the deletion or functional silencing of a sufficient proportion of HBV-specific T cell clones (Milich 2016).

The second phase, historically referred to as the immune clearance phase or HBeAg-positive chronic hepatitis, most commonly occurs during the third or fourth decade of life in

individuals infected with HBV early in life. During this phase, activation of T cell-mediated immune responses against infected hepatocytes results in cytolytic release of alanine aminotransferase (ALT) and a reduction in serum HBV DNA levels. The intensity of the immune response, however, may fluctuate over time, leading to variable ALT and HBV DNA levels. Liver injury associated with hepatitis flares can result in varying degrees of fibrosis and cirrhosis. The duration of this phase is highly variable and typically concludes with a decline in HBV DNA levels and seroconversion of HBeAg to anti-HBe positivity (Yuen et al. 2018).

The immune residual phase or HBeAg-negative chronic infection, is characterized by the presence of anti-HBe. These individuals, historically referred to as inactive carriers, generally exhibit near-normal serum aminotransferase levels and HBV DNA concentrations below 2,000 IU/mL. Early seroconversion to anti-HBe, occurring before significant hepatic fibrosis develops and accompanied by persistently low viral replication and minimal liver inflammation, is associated with a favorable prognosis. However, in general, HBeAg-negative patients are older, and if patients still have moderate or high levels of viral replication, they are at greater risk of progressive liver damage and more rapid development of cirrhosis (Yuen et al. 2018). Notably, in a significant proportion of patients from Asian and Mediterranean regions, HBeAg seroconversion is associated with the emergence of precore and/or core promoter mutations, resulting in the selection of HBV variants that do not express HBeAg (Yuan et al. 2007). Although 67–80% of patients remain in this phase, viral replication can occasionally reactivate, resulting in fluctuations of both HBV DNA levels and serum ALT. This phase is referred to as residual disease flares or HBeAg-negative chronic hepatitis (L. S. Y. Tang et al. 2018).

At any stage during chronic HBV infection, spontaneous immunologic clearance may occur, defined as the loss of HBsAg with or without seroconversion to anti-HBs, accompanied by undetectable HBV DNA in peripheral blood. Immunologic clearance is associated with improved survival and a reduced risk of liver failure and hepatocellular carcinoma, and in the absence of antiviral therapy, it occurs at an annual rate of 0.5% to 2% (C. Chen and Yang 2011).

Three categories of cure for chronic HBV infection have been defined: virological, functional (also referred to as immunologic), and partial. Virological cure is defined as the complete eradication of HBV DNA from the blood and liver while serological tests for anti-HBc remain positive, with or without anti-HBs. However, covalently closed circular DNA cannot be fully eliminated from hepatocytes, rendering virological cure theoretical and currently unattainable (L. S. Y. Tang et al. 2018).

Functional cure is defined by the loss of HBsAg combined with undetectable HBV DNA in peripheral blood, sustained indefinitely after a finite course of therapy. Unlike virological cure, functional cure is achievable, although it occurs in only 3% to 11% of patients treated with interferon therapy (Terrault et al. 2016) (Buster et al. 2008).

Partial cure is characterized by HBeAg loss, which is associated with a decreased level of HBV DNA (termed HBV DNA suppression) and normalization of ALT levels, indicative of reduced hepatic inflammation. In this context, HBV DNA remains low (<2,000 IU/mL) or undetectable after treatment cessation, while HBsAg remains detectable, defining the partial cure state (Lok et al. 2017).

2.1.4.4 – Pathogenesis

Liver tumor is currently the fourth leading cause of death by cancer in the world, with 782,000 deaths per year. In particular, Hepatocellular carcinoma (HCC) represents 90% of all primary liver tumors, and it is related in 50% of cases to HBV (Bray et al. 2018). In patients infected with HBV, the progression of liver disease is driven by the persistent and ineffective immune responses aimed at clearing the virus, which result in ongoing chronic liver injury (Shirvani-Dastgerdi et al. 2016).

Genomic alterations and transcriptomic dysregulation observed in HCC are closely linked to underlying risk factors. A recent study compared the mutational and transcriptomic profiles of HBV-related HCC with those of HCC arising from other etiologies (Zucman-Rossi et al. 2015). The results indicate that, in HBV-related hepatocellular carcinoma, TP53 mutations are more frequent than in other etiologies, representing a major mechanism of cell cycle pathway alteration. As a key regulator of chromosomal stability, the loss of TP53 function constitutes an important determinant in HCC development (Amaddeo et al. 2015). Another genomic feature of HBV-related HCC is the presence of

frequent viral integrations in genes involved in carcinogenesis, such as TERT, MLL4, and CCNE1. Moreover, the action of HBxAg and other viral proteins such as HBs contributes to the development of HCC (Levrero and Zucman-Rossi 2016).

Therefore, the direct oncogenic factors include insertional mutagenesis, chromosomal instability, and viral protein activity. They are responsible for approximately 10–30% of HBV-associated hepatocellular carcinomas (HCC) arising in a non-cirrhotic liver. However, HBV can also promote tumorigenesis through indirect mechanisms. These indirect oncogenic effects are primarily driven by chronic inflammation leading to liver cirrhosis, which plays a pivotal role in hepatocarcinogenesis, as up to 85% of HCC cases develop on a cirrhotic background (Llovet et al. 2016) (*Figure 16*).

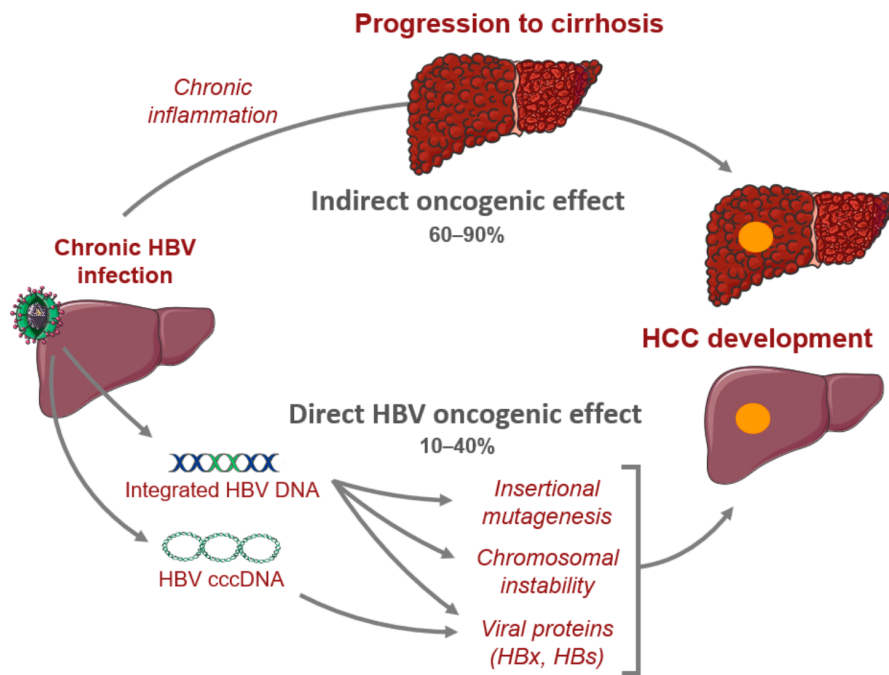


Figure 16: Oncogenic mechanisms related to hepatitis B virus (Péneau et al. 2021)

2.1.5 – Prevention and treatment

2.1.5.1 – Vaccine

The age at which HBV infection occurs is a critical determinant of the risk of chronicity, exceeding 90% in infants and children under one year of age, approximately 30% in those aged one to five years, and only 0–2% in adults. Consequently, the majority of chronic HBV

infections are acquired through mother-to-child transmission at birth or during early childhood (Stevens et al. 1975).

Safe and effective vaccines are available to prevent HBV infection and are the key of primary prevention. The implementation of childhood vaccination programs has been pivotal in reducing the prevalence of hepatitis B among children (Ott et al. 2012). The WHO advocates universal HBV vaccination, recommending that all newborns receive the first dose within 24 hours of birth. This initial dose, given as part of the Expanded Programme on Immunization (EPI), is followed by two or three additional doses, according to national guidelines and local prevalence rates (Yuen et al. 2018).

The first hepatitis B vaccines, commercially available since 1982, were derived from plasma. These vaccines were developed by the American microbiologist Maurice Hilleman, who produced them by isolating subviral particles of HBsAg from the plasma of asymptomatic individuals chronically infected with HBV. The pooled plasma was purified, and any residual infectious particles were subsequently inactivated through various combinations of urea, pepsin, formaldehyde, and heat treatments (Hilleman et al. 1975). These first vaccines were commercialized under the trade names *Heptavax B* (Merck) and *Hevac B* (Institut Pasteur), and were primarily administered to high-risk groups, which represented the main target population of immunization programs at that time. However, a broad vaccination coverage failed due to the elevated costs of vaccination and the absence of comprehensive global immunization policies (Pattyn et al. 2021). In 1986, the first genetically engineered hepatitis B vaccine was developed through the use of recombinant hepatitis B surface antigen (HBsAg), resulting in a second generation of HBV vaccines that fully replaced plasma-derived formulations (Shouval 2003). The application of recombinant DNA technology to express HBsAg in yeast, and subsequently in mammalian cells, enabled the large-scale production of vaccine material. The expressed HBsAg spontaneously assembles into virus-like particles (VLPs), and its administration elicits protective antiviral immunity against HBV infection. The development of recombinant hepatitis B vaccines represents a landmark achievement in vaccinology, as they were the first vaccines to be based on VLP technology (Pattyn et al. 2021). Recombinant hepatitis B vaccines are characterized by a high degree of immunogenicity. Multiple immunization schedules have been demonstrated to induce seroprotective antibody levels exceeding 95% in healthy infants, children, and young adults (Vesikari and Van Damme 2017). Vaccines derived from mammalian cell cultures contain

glycosylated pre-S1 and pre-S2 proteins in addition to the major HBsAg protein. By targeting epitopes beyond the HBsAg S region, these formulations, together with novel adjuvanted vaccine candidates under development, have shown enhanced immunogenicity compared with second-generation vaccines (Shouval et al. 2015).

2.1.5.2 – Antiviral therapy

In chronic HBV infection, treatment indications are determined by serum HBV DNA levels, alanine aminotransferase (ALT) concentrations, and the severity of liver disease. Treatment indications are similar for both HBeAg-positive and HBeAg-negative cases, with treatment typically recommended for individuals with HBV DNA levels exceeding 2,000 IU/mL or with elevated ALT levels over the upper limit of normal (ULN) (Lampertico et al. 2017). HBV treatment is typically aimed at achieving a functional cure with a normal range of ALT value, histological improvement, and prevention of the complications of liver disease, such as cirrhosis, liver failure, and HCC. The functional cure is represented by HBsAg seroclearance; nevertheless, HBsAg seroclearance is rarely achieved with currently available anti-HBV therapies. Therefore, a more realistic therapeutic endpoint is the induction of sustained virological remission, defined by undetectable HBV DNA levels and HBeAg seroconversion. In contrast, the management of acute HBV infection is primarily supportive, and antiviral therapy is generally not recommended except in cases of fulminant hepatic failure (G.-A. Kim et al. 2014).

Therapeutic agents approved by the U.S. Food and Drug Administration (FDA), the European Medicines Agency, and regulatory authorities in many Asian countries are broadly categorized into immunomodulatory and antiviral agents. Immunomodulatory agents, such as interferons, enhance the host immune response and may achieve sustained viral control, but their efficacy is limited, and adverse effects are frequent. Conversely, antiviral agents, directly inhibit viral replication and are well tolerated, although they usually require long-term treatment and may lead to resistance.

The immunomodulatory group comprises conventional IFN α -2b and pegylated IFN α -2a, whereas the antiviral group consists of nucleos(t)ide analogues (NUCs), including lamivudine, adefovir dipivoxil, entecavir, telbivudine, tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide (TAF) (*Figure 17*).

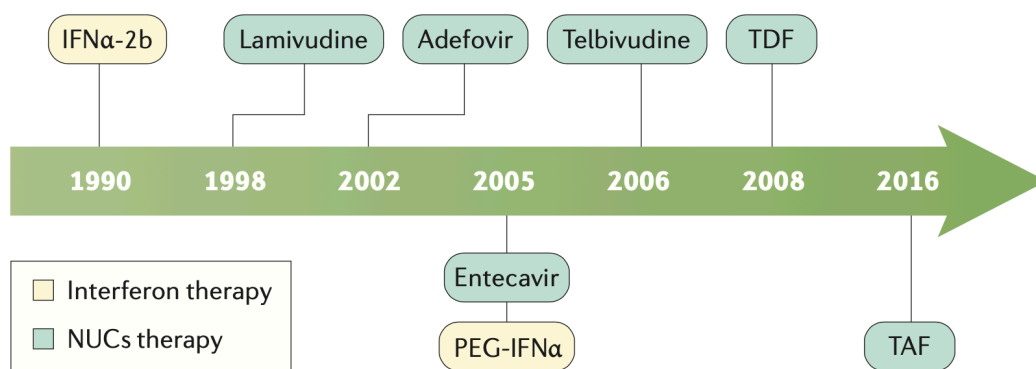


Figure 17: Approved treatment agents for chronic HBV infection (Yuen et al. 2018)

The NUCs inhibit the RNA-dependent DNA polymerase reverse transcriptase. Among the six NUCs approved by the FDA, entecavir, TDF, and TAF are recommended as first-line therapeutic agents owing to their high antiviral potency and low rates of resistance. Entecavir is generally well tolerated, with negligible adverse effects, and is considered an optimal choice for treatment-naïve patients. However, in individuals previously treated with lamivudine who have developed resistance, entecavir is less suitable. This is because lamivudine-resistant patients already harbor two of the three mutations required for entecavir resistance, leading to a cumulative resistance rate of approximately 51% within five years of therapy (Tenney et al. 2009). In such cases, TDF and TAF represent the preferred treatment options. Compared with TDF, TAF achieves higher intrahepatic concentrations of tenofovir, thereby allowing for lower dosing to obtain equivalent viral suppression. This pharmacokinetic advantage results in fewer off-target effects with TAF (Murakami et al. 2015).

NUCs exert a profound effect on viral suppression when administered continuously. Indeed, treatment with NUCs can achieve sustained suppression of HBV DNA to very low, often undetectable levels in all patients (Lai and Yuen 2008), and for the majority of the patients, a biochemical response as normalization of ALT levels is observed (Marcellin et al. 2013). Long-term follow-up studies of sustained NUC therapy have demonstrated that early initiation of treatment markedly reduces the risk of cirrhosis, leading to the near elimination of liver failure among treated patients with chronic HBV infection. Furthermore, such therapy significantly decreases the incidence of HCC (Lai and Yuen 2013). Overall, adverse events associated with long-term NUC therapy are considered minimal, and these therapeutic benefits are independent of HBV genotype.

The precise mechanism by which interferons exert their antiviral effect is still unclear. However, it is believed that they act both through direct antiviral activity, including perturbations of covalently closed circular DNA, the degradation of viral messenger RNA, and the inhibition of viral DNA replication, as well as through host immunomodulatory effects, such as the enhancement of the immune response against infected hepatocytes and the facilitation of viral clearance (Rijckborst and Janssen 2010). Pegylated interferon- α (PEG-IFN α) is the injectable formulation and is administered subcutaneously once weekly in selected patient populations with appropriate treatment indications. In particular, polyethylene glycol (PEG) is conjugated to interferon to improve its pharmacokinetic properties, as pegylation increases the molecular size, reduces renal clearance, and prolongs the serum half-life. A 48-week course of PEG-IFN α therapy results in a sustained off-treatment virological response in approximately 30% of treated patients (Marcellin et al. 2004). The ideal therapeutic goal is the functional cure and thus the HBsAg loss, although this outcome is achieved in only about 10% of patients. Prolonged treatment durations may increase response rates but are frequently limited by tolerability issues (Lampertico et al. 2013). In fact, adverse events of PEG-IFN α are common and necessitate close monitoring for clinical symptoms such as malaise, fever, thyrotoxicosis, and depression, as well as regular laboratory assessment of hematologic parameters, liver biochemistry, and thyroid function.

Given the modest overall efficacy and the substantial risk of adverse effects, PEG-IFN α therapy should be individualized and reserved for patients with the highest predicted likelihood of response. In HBeAg-positive patients, PEG-IFN α is a reasonable first-line option for individuals infected with HBV genotypes A or B, which are more responsive to interferon-based therapy (Buster et al. 2009a). In HBeAg-negative patients, pretreatment factors associated with a favorable response at 24 weeks include younger age, female sex, elevated baseline ALT levels, and low HBV DNA levels (Bonino et al. 2007).

Studies investigating combination therapy have sought to determine whether the concomitant use of different agents can enhance the rate of HBsAg seroclearance. In one trial involving 740 treatment-naïve patients infected with genotypes A or B, the combination of PEG-IFN α with TDF achieved a higher rate of HBsAg seroclearance: 9.1% with TDF plus 48 weeks of PEG-IFN α , 2.8% with TDF plus 16 weeks of PEG-IFN α , 0% with 120

weeks of TDF monotherapy, and 2.8% with 48 weeks of PEG-IFN α monotherapy. These findings indicate that combination therapy is superior to either TDF or PEG-IFN α alone (Marcellin et al. 2016). By contrast, another study conducted in NUC-treated, HBeAg-negative patients with undetectable HBV DNA found that the addition of 48 weeks of PEG-IFN α did not significantly increase HBsAg seroclearance compared with NUC therapy alone (Bourlière et al. 2017). These discrepant outcomes suggest that the benefit of combining PEG-IFN α with NUCs may be restricted to treatment-naïve individuals, particularly those infected with genotypes A or B. Anyway, combination therapy is not routinely recommended.

2.2- Interferons

2.2.1- Classification and Signaling Mechanisms of Interferons

Interferons (IFNs) constitute one of the innate defense mechanisms of the human organism against the invasion of external agents, including viruses, microbes, and tumor cells. By binding to specific receptors, they initiate signal transduction pathways that induce the expression of a wide array of genes, which are now recognized as being involved not only in antiviral responses but also in immunomodulatory and antiproliferative activities (De Andrea et al. 2002). IFNs are classified into three major types: type I, type II, and type III. A common feature across all IFNs is their capacity to elicit antiviral responses through the production of interferon-stimulated genes (ISGs), whose products inhibit various stages of viral replication. Type I IFNs were first identified as soluble factors mediating viral interference, defined as resistance to viral infection induced by a prior infection. Following two decades of investigation, type I IFN genes were cloned, sequenced, and formally established as a related gene family (Levy 2001). Type I IFNs are predominantly expressed by innate immune cells. Type II IFN is represented by a single gene product, IFN- γ , which was identified for its antiviral activity induced by activated immune cells, particularly natural killer (NK) cells and T lymphocytes. Type III IFNs, also referred to as IFN- λ , display a restricted tissue distribution and are not highly expressed in hematopoietic cells. Their activity is primarily localized to epithelial surfaces (Negishi et al. 2018).

The type I IFN superfamily encompasses four distinct families: IFN- α , IFN- β , IFN- ϵ , IFN- κ , and IFN- ω . Among these, the α and β families are the most extensively characterized. The α family is the only one comprising multiple subtypes, with 13 distinct IFNs identified ($\alpha 1$,

$\alpha 2, \alpha 4, \alpha 5, \alpha 6, \alpha 7, \alpha 8, \alpha 10, \alpha 13, \alpha 14, \alpha 16, \alpha 17$, and $\alpha 21$). A single member, IFN- γ , represents Type II IFN. The type III superfamily consists of a single family, IFN- λ , which includes four subtypes ($\lambda 1, \lambda 2, \lambda 3$, and $\lambda 4$) (Walter 2020). Type I IFNs exert their activity through the IFNAR1–IFNAR2 receptor complex. Type II IFN- γ signals via the IFNGR1–IFNGR2 receptor complex, whereas type III IFNs act through the IFN- λ R1–IL-10R2 receptor complex. In each case, the receptor heterodimer is composed of a high-affinity binding chain (such as IFNAR2, IFNGR1, or IFN- λ R1) paired with a low-affinity binding chain (IFNAR1, IFNGR2, or IL-10R2) (Deshpande et al. 2013).

Upon binding to their receptors, type I IFNs signaling involves the recruitment of the Janus kinases TYK2 and JAK1. Activation of these kinases induces tyrosine phosphorylation of STAT1 and STAT2, leading to the formation of the trimeric transcription factor called IFN-stimulated gene factor 3 (ISGF3), consisting of STAT1, STAT2, and IRF9. Upon nuclear translocation, ISGF3 binds to IFN-stimulated response elements (ISREs) within the promoters of interferon-stimulated genes (ISGs), thereby initiating their transcription (Ivashkiv and Donlin 2014).

Type II IFN (IFN- γ) signal transduction is mediated through a distinct receptor complex composed of IFNGR1 and IFNGR2, which associate with JAK1 and JAK2, respectively. The subsequent activation of these kinases promotes STAT1 phosphorylation, homodimerization, and nuclear translocation, where STAT1 dimers bind to gamma-activated sequence (GAS) elements. Furthermore, ISGF3 can also be weakly activated by IFN- γ , although not with the same efficiency as type I IFNs (Takaoka et al., 2000).

Type III IFNs are the least well characterized, but they activate the same JAK–STAT signaling pathway as type I IFNs, leading to ISGF3 formation and induction of ISGs (Lazear et al. 2015) (*Figure 18*).

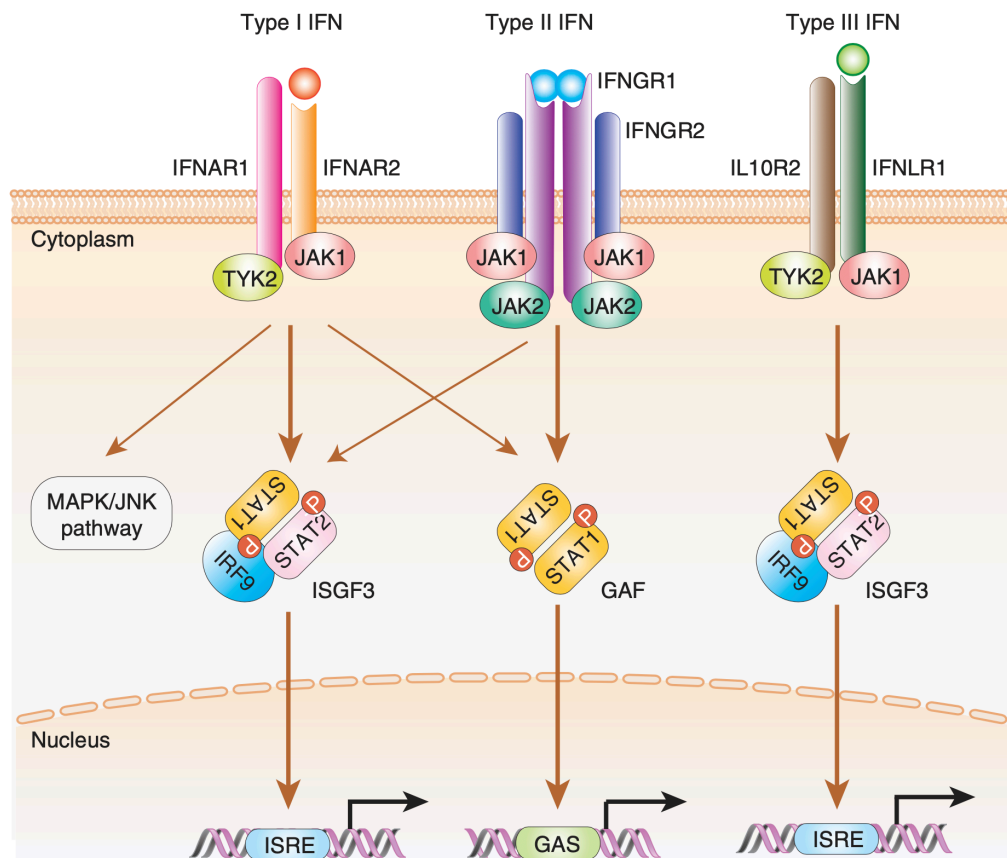


Figure 18: Signal transduction pathways for the three types of IFN (Negishi et al. 2018)

The interferon regulatory factors (IRFs) are a key component of IFN's response and are strictly related to the modulation of IFNs. IRFs consist of nine members: IRF1, IRF2, IRF3, IRF4 (also known as PIP/LSIRF/ICSAT), IRF5, IRF6, IRF7, IRF8, and IRF9. Collectively, these transcription factors orchestrate the regulation of gene expression programs that underlie the initiation and fine-tuning of IFN-mediated responses (Mancino and Natoli 2016).

2.2.2- IFNs production

The production of IFN type I is initiated through two principal recognition pathways: Toll-like receptors (TLRs) and other innate immune sensing mechanisms.

TLRs were the first pattern recognition receptors (PRRs) identified and are essential for detecting a wide range of pathogen-associated molecular patterns (PAMPs) and, in some cases, damage-associated molecular patterns (DAMPs). Their activation induces the

transcription of type I IFNs and proinflammatory cytokines, thereby triggering immune responses (Medzhitov 2001).

Different TLRs exhibit distinct ligand specificities and cellular localizations. For instance, TLR3 is predominantly localized in endosomes, where it recognizes viral double-stranded RNA or self-RNA released from dying cells (Rudd et al. 2006). By contrast, TLR4 resides at the plasma membrane and senses diverse bacterial PAMPs as well as DAMPs from necrotic cells (Andersson and Tracey 2011).

Upon ligand binding, TLRs initiate signaling cascades by recruiting adaptor proteins, including myeloid differentiation primary-response protein 88 (MyD88) and/or Toll/IL-1 receptor (TIR) domain-containing adaptor inducing IFN- β (TRIF). These adaptors subsequently activate IRFs and other transcription factors, culminating in the transcription of genes required for IFN-I production (*Figure 19*).

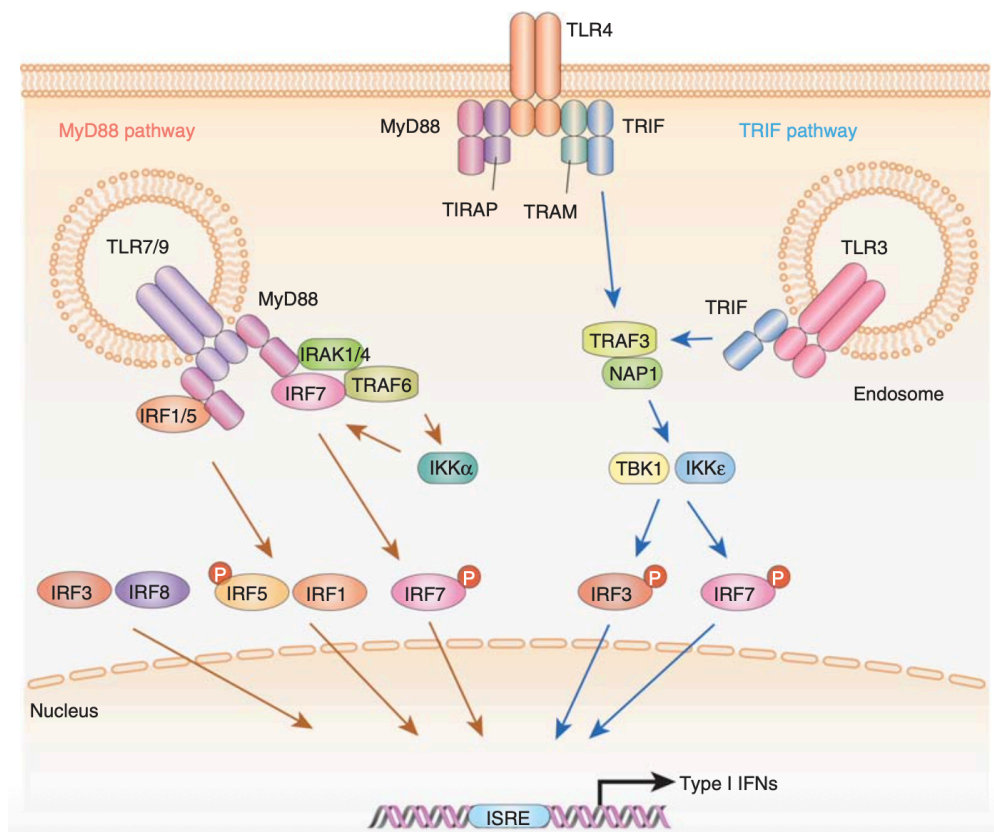


Figure 19: TLR-mediated type I IFN gene induction by IRFs (Negishi et al. 2018)

Cytosolic detection of viral RNA by innate immunity represents another major pathway for type I interferon (IFN-I) induction and is mediated primarily by two RNA helicases: retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5).

These receptors recognize distinct viral RNA signatures, such as uncapped 5'-triphosphate RNA and double-stranded RNA (dsRNA). Both helicases share a conserved carboxy-terminal DExD/H-box RNA helicase domain responsible for RNA sensing, as well as two amino-terminal caspase activation and recruitment domains (CARDs), which initiate downstream signaling (Yoneyama et al. 2005)

Signal transduction from RIG-I and MDA5 is mediated by the adaptor protein mitochondrial antiviral signaling protein (MAVS). Once activated, MAVS recruits key transcription factors, most notably IRF3 and IRF7, which are indispensable for the RIG-I/MDA5-dependent IFN-I gene induction pathway. Alternatively, MAVS can also activate NF- κ B, thereby contributing to the broader antiviral transcriptional program. Although additional IRFs may participate in this signaling cascade, the outcome of RIG-I/MDA5–MAVS activation is the transcription of type I IFN genes, ensuring a rapid and robust antiviral response (Tamura et al. 2008) (*Figure 20*).

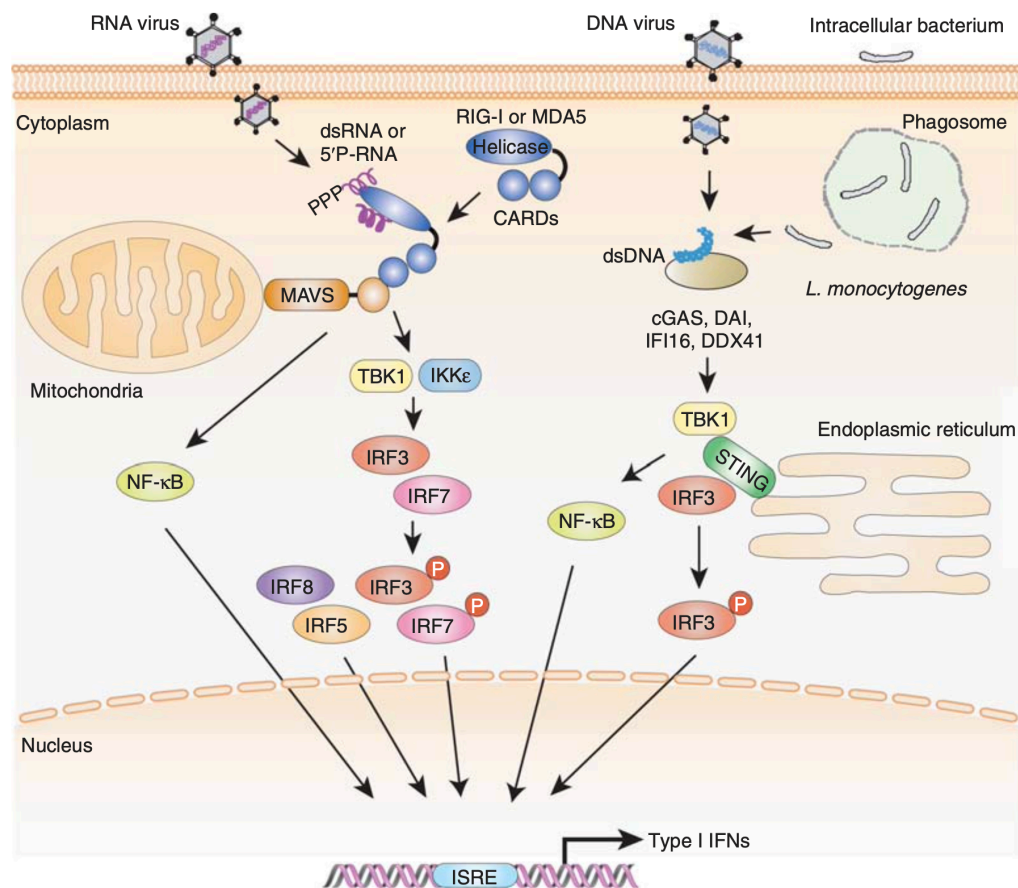


Figure 20: type I IFN gene induction by IRFs on innate recognition of cytosolic nucleic acids (Negishi et al. 2018)

In addition to RNA-sensing mechanisms, significant attention has been directed toward cytosolic DNA recognition systems, as they are capable of eliciting both protective and pathological immune responses. The discovery of cytosolic DNA sensors capable of triggering innate immune signaling, particularly the induction of type I IFN genes, has greatly expanded our understanding of antiviral defense. The first such sensor identified was the DNA-dependent activator of IRFs (DAI) (Takaoka et al. 2007). Subsequent studies have revealed additional sensors with complementary roles, including cyclic GMP–AMP synthase (cGAS), IFN-inducible protein 16 (IFI16), and DEAD-box helicase 41 (DDX41) (Negishi et al. 2018)(Ishikawa et al., 2009). Central to this pathway is the adaptor protein STING, which is essential for dsDNA-mediated IFN induction. STING functions as a recruiter for TBK1, which phosphorylates IRF3, leading to its activation and subsequent induction of type I IFN genes (*Figure 20*).

Production of type II IFN, known as IFN- γ , was initially attributed exclusively to CD4⁺ T helper 1 (Th1) lymphocytes, CD8⁺ cytotoxic T lymphocytes, and natural killer (NK) cells. More recent evidence, however, indicates that additional immune cell types, including B cells, NK T cells, dendritic cells, and macrophages, are also capable of secreting IFN- γ . The promoter region of the IFN- γ gene contains binding sites for multiple transcription factors, such as AP-1, CREB/ATF, NFAT, NF- κ B, T-bet, Eomes, and STATs, but notably lacks IRF motifs. Activation of IFN- γ gene expression is typically induced by T-cell receptor engagement or by cytokine stimulation, including type I IFNs, interleukin-12 (IL-12), and interleukin-18 (IL-18), which converge on these transcriptional regulators (Schoenborn and Wilson 2007).

Similar to type I IFNs, type III IFNs are induced following the recognition of diverse viral PAMPs. Nucleic acid-sensing PRRs, including TLR3, TLR7/8, TLR9, and cGAS, have been implicated in this process, although the precise molecular mechanisms remain incompletely defined. The RIG-I-like receptor (RLR)-mediated induction of type III IFNs has been studied in detail and relies on signaling pathways largely overlapping with those of type I IFNs (Lazear et al. 2015). Despite these shared mechanisms, important differences exist in transcriptional regulation. In particular, NF- κ B contributes but is not essential for

type I IFN gene expression, but it plays a more critical role in type III IFN transcription (Iversen and Paludan 2010). Another distinction lies in the involvement of IRF1, which participates in the induction of type III, but not type I, IFNs (Odendall et al. 2014).

2.2.3 - Effects of Interferon on HBV Infection *in vitro*

The efficacy of interferon (IFN) in *in vitro* studies has yielded conflicting results, largely influenced by the specific experimental protocols employed, the HBV genotype under investigation, and the intrinsic sensitivity of the chosen *in vitro* model to IFN. Clinical data indicate that, compared with genotypes C and D, patients infected with genotypes A and B achieve higher rates of HBeAg seroconversion following IFN- α treatment (Buster et al. 2009b). Among the various genotypes, genotype D3 isolated from Europe represents the most extensively studied in cell culture, as it constitutes the best-characterized molecular clone of HBV. Although this clone and its derivative cell lines producing infectious virus have proven indispensable for elucidating HBV biology, they may not accurately reflect the broader diversity of viral genotypes circulating worldwide. However, conflicting data exist regarding the specific role of HBV genotypes in IFN- α response (Sonneveld et al. 2012).

As regards primary human hepatocytes, different HBV genotypes were tested and subsequently treated with IFN- α 2a to evaluate antiviral effects. Distinct HBV genotypes displayed variable profiles of HBsAg, HBeAg, and HBV DNA production *in vitro*. In mPHH cells, genotype B2 showed the highest infectivity and replication efficiency, while D3 and D4 displayed similar replication profiles. Antigen production varied across genotypes, with B2 again producing the highest levels. In contrast, genotype C1 exhibited markedly lower infectivity, requiring a higher inoculum to establish infection. Overall, this work demonstrates that IFN- α 2a exerts potent antiviral effects across all tested genotypes in primary hepatocytes, with some variability in response likely reflecting differences in genotype-specific infectivity (M. Zhang et al. 2021).

Several studies in immortalized cell lines have demonstrated that the antiviral effect of IFN is less pronounced compared to primary hepatocytes. In HepG2-NTCP cells, for instance, HBV genotype A2, B5, C2, D, and D3 showed only a limited reduction in replication following IFN- α 2a treatment, with measurable effects observed exclusively at high

concentrations. Overall, findings from cell culture–based HBV infection models indicate that HBV sensitivity to IFN- α 2a in hepatocytes is determined more by the intrinsic cellular IFN response than by viral genotype. Importantly, the study also showed that while IFN- α 2a displayed limited efficacy in HepG2-NTCP cells, the IFN- α 14 subtype exhibited markedly enhanced antiviral activity (Shen et al. 2018). Among the 13 human IFN- α subtypes, IFN- α 2 has been the most extensively studied and has served as the standard therapy for chronic hepatitis B (CHB). IFN- α 14 was identified as a highly potent IFN- α subtype with superior antiviral activity against HBV *in vitro* and *in vivo*. Specifically, IFN- α 14 strongly suppresses HBV antigen production and cccDNA transcription, thereby contributing to the development of therapeutic strategies aimed at achieving HBsAg loss and durable cccDNA silencing, two critical milestones toward a functional cure for HBV (Revill et al. 2019). Mechanistically, the potent antiviral activity of IFN- α 14 appears to be mediated by a strong and specific interaction with IFNAR1. Supporting this, an engineered IFN- α 2 variant containing the IFN- α 14–specific IFNAR1 binding sequence (IFN- α 2–EIFK) displayed antiviral efficacy comparable to IFN- α 14. Moreover, IFN- α 14 exhibited IFN- γ –like activity, inducing expression of a distinct subset of genes typically regulated by IFN- γ . While high doses of IFN- α 2 could also trigger an IFN- γ –like response, this was associated with increased cytotoxicity, underscoring a potential therapeutic advantage of IFN- α 14 (J. Chen et al. 2021).

Considering type III interferons (IFN- λ) in comparison to type I IFNs across different cellular systems, several studies have evaluated their effects on HBV cccDNA in infected cells. Treatment with IFNs, both type I and III, led to the upregulation of the cytidine deaminase APOBEC3A, resulting in cccDNA deamination and partial cccDNA loss. Although cccDNA reduction was comparable among IFNs, IFN- λ 1 exerted the most favorable antiviral activity at relatively low doses. Moreover, IFN- λ induced a more sustained ISG response compared to IFN- α , consistent with prior data showing that IFN- α –mediated antiviral states become refractory after approximately three days of treatment (Makowska et al. 2011). These findings suggest that IFN- λ may offer therapeutic advantages over IFN- α 2, particularly with respect to promoting cccDNA destabilization and silencing. However, it should be noted that the antiviral effects of IFN- λ on viral antigen expression were largely comparable to those of IFN- α , indicating that their advantage may be limited primarily to cccDNA regulation rather than antigen suppression (Bockmann et al. 2019).

2.3 - CRISPR-Cas9 Screening

2.3.1 – The CRISPR-Cas9 System

Scientists have identified a novel microbial defense system that protects against viral and mobile genetic elements. Among these mechanisms, the CRISPR/Cas system in bacteria and archaea functions by integrating fragments of invading DNA into the host genome, thereby creating a molecular memory of past infections (Horvath and Barrangou 2010).

The CRISPR/Cas system provides adaptive immunity against invading viruses and foreign genetic elements through three sequential stages: adaptation, crRNA biogenesis, and interference (Deltcheva et al. 2011) (*Figure 21*). During the adaptation stage, the entry of exogenous genetic material into bacterial cells activates the Cas1–Cas2 complex, which excises fragments of the foreign DNA and integrates them into the host CRISPR array as new spacers. In the subsequent biogenesis stage, the CRISPR locus is transcribed into a precursor crRNA (pre-crRNA), which is then processed by Cas nucleases into mature crRNAs. These crRNAs assemble with Cas proteins to form effector complexes. In the interference stage, mature crRNAs associate with Cas proteins to form effector complexes. When a sequence of foreign genetic material matches a CRISPR spacer, the corresponding crRNA guides the complex to the invading strand, which is then cleaved by the Cas nuclease. This mechanism prevents the bacterium from being reinfected by viruses or genetic invaders (Barrangou et al. 2007).

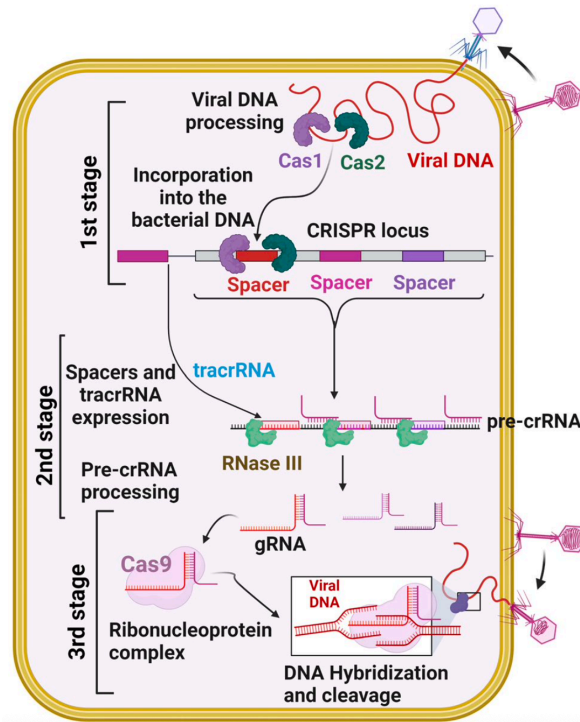


Figure 21: CRISPR/Cas adaptive immunity system (Hillary and Ceasar 2023)

Genome editing has emerged as a transformative field in molecular biology, with the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) system rapidly establishing itself as the leading technology, inspired by the natural CRISPR/Cas immune mechanism (Rao et al. 2022). In general, Genome editing relies on programmable sequence-specific endonucleases that generate targeted single-stranded breaks (SSBs) or double-stranded breaks (DSBs), which are subsequently repaired by endogenous cellular mechanisms. Two major DNA repair pathways operate in this context: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Kass and Jasin 2010).

A major breakthrough came in 2012 with the establishment of the CRISPR-Cas system as a genome-editing tool. Originally discovered as an adaptive immune mechanism in bacteria and archaea to defend against phages, the CRISPR-Cas system was rapidly adopted by the scientific community due to its simplicity of design, cost-effectiveness, and versatility as an RNA-guided DNA-targeting technology (Jinek et al. 2012).

Mechanistically, the CRISPR system is divided into two main classes and six types (I–VI). Types I, II, and V primarily target DNA, type VI cleaves RNA, type III is capable of cleaving both DNA and RNA, while the activity of type IV remains poorly defined (F. Wang et al. 2019). Class 1 systems require multiple effector proteins for RNA-guided target cleavage,

whereas Class 2 systems rely on a single RNA-guided endonuclease. The latter includes type II Cas9 and type V Cas12a, which are particularly well-suited for genome editing in eukaryotic cells and have become the most widely employed platforms (Koonin et al. 2017).

Cas9 is an endonuclease associated with the CRISPR adaptive immune system of *Streptococcus pyogenes*, commonly referred to as SpyCas9. SpyCas9 is a large multifunctional protein of 1368 amino acids that functions as a DNA nuclease in both natural and engineered CRISPR/Cas systems (Hillary and Ceasar 2023).

Structurally, Cas9 consists of six distinct domains: (1) the Recognition lobe (REC I), primarily responsible for gRNA binding; (2) REC II, whose precise function remains unclear; (3) an arginine-rich bridge helix that initiates cleavage upon target binding; (4) the PAM-interacting domain, which confers protospacer adjacent motif (PAM) specificity; (5) the HNH nuclease domain; and (6) the RuvC nuclease domain, both of which mediate target DNA cleavage (Nishimasu et al. 2014) (Figure 22).

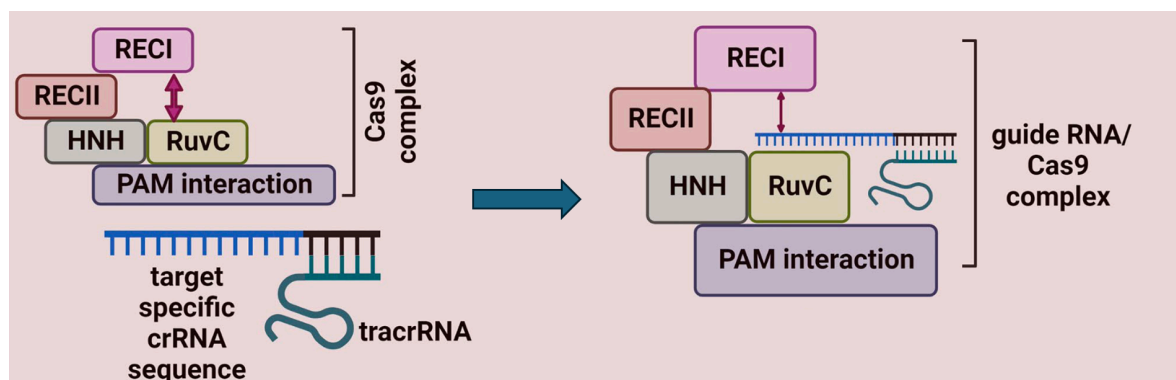


Figure 22: The Cas9 protein complex with its six domains (Hillary and Ceasar 2023)

The mature crRNA contains the DNA target sequence, while the trans-activating RNA (tracrRNA) is a structural support. When the crRNA base-paired with the tracrRNA, it forms a dual-RNA structure that guides the Cas9 protein to introduce double-stranded breaks (DSBs) at specific target DNA sites. This natural dual system (tracrRNA-crRNA) can be engineered into a single chimeric guide RNA (sgRNA), which retains the same functionality and directs Cas9 to cleave double-stranded DNA in a sequence-specific manner. In its native state, Cas9 remains inactive in the absence of guide RNA (sgRNA). The sgRNA is designed with a 5' region complementary to the target sequence, enabling specific base pairing. Upon binding to Cas9, the sgRNA induces conformational rearrangements that activate the protein.

The active Cas9–sgRNA complex then scans the genome for target sites, recognizing sequences adjacent to the PAM motif (5'-NGG-3'). Cleavage occurs three base pairs upstream of the PAM: the HNH domain cleaves the DNA strand complementary to the sgRNA, whereas the RuvC domain cleaves the non-complementary strand (Jinek et al. 2012) (*Figure 23*).

DSB repair occurs primarily through two pathways: NHEJ and HDR. NHEJ, characterized by its error-prone nature, often introduces small insertions or deletions at the DSB site. In contrast, HDR utilizes a homologous DNA template to enable precise sequence modification, but it also induces undesirable mutations. These mutations result in the failure of protein translation and thus the knock out of the gene of interest (Aljabali et al. 2024).

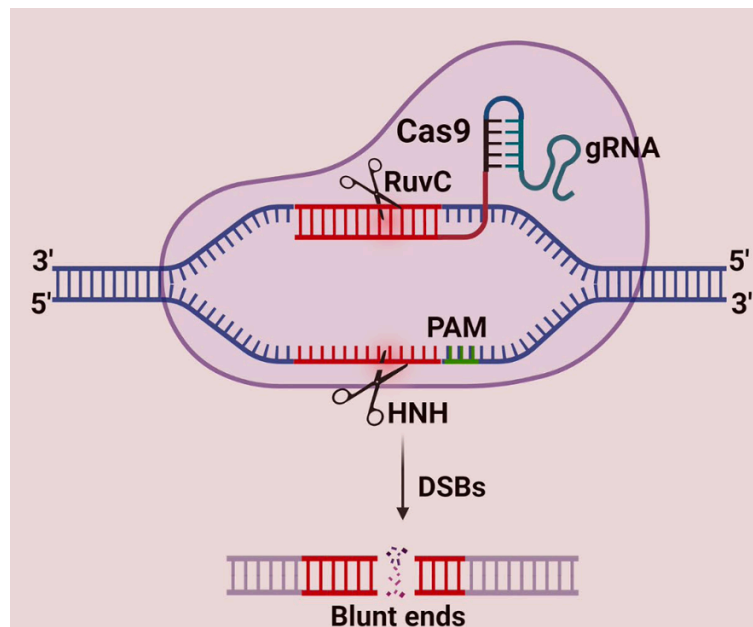


Figure 23: CRISPR/Cas9 mechanism (Hillary and Ceasar 2023)

A major advantage of the CRISPR/Cas9 system lies in its simplicity of design and higher efficiency compared to earlier genome-editing platforms such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Another key benefit is its capacity for multiplexed genome editing, which can be achieved by designing and introducing multiple sequence-specific gRNAs simultaneously (Jinek et al. 2012).

Nevertheless, despite these advances, the CRISPR/Cas9 system presents several limitations and raises concerns regarding the risks associated with genome editing. For instance, sgRNAs that guide Cas9 to cleave double-stranded (dsDNA) or single-stranded DNA (ssDNA) have been shown to induce both on-target and off-target mutations in host

genomes. To mitigate such off-target effects, engineered variants such as nickase Cas9 (nCas9) have been developed. Unlike wild-type Cas9, which generates double-stranded breaks, nCas9 introduces single-strand nicks, thereby reducing the frequency of unwanted edits. However, off-target effects cannot be completely eliminated with nCas9, highlighting the need for further refinement and development of next-generation Cas9 variants (Rodriguez-Rodriguez et al. 2019).

2.3.2 – Functional genomic screening

Genetic screening represents a powerful strategy for elucidating genes, pathways, and molecular mechanisms that underlie specific phenotypes or biological processes. CRISPR-based screening has shown the efficiency and versatility of CRISPR–Cas genome editing, rapidly becoming a widely adopted and highly productive tool for biological discovery across different applications (Shalem et al. 2015).

Loss-of-function screens rely on the stable inactivation of genes, either through knockdown or complete knockout. Early RNAi-based approaches provided important insights into virus–host interactions (Ramage and Cherry 2015), although the partial nature of gene silencing limited them. Advances in genome editing technologies now allow the full ablation of gene expression, representing a major advantage of CRISPR-based knockout strategies. The specific features of each loss-of-function approach are summarized in *Figure 24*.

	CRISPR–Cas	RNAi
Mechanism	Induced DSBs lead to error-prone NHEJ and, frequently, frameshift mutations	siRNAs or shRNAs bind to target mRNAs, which leads to their cleavage and degradation
Phenotype	Complete knockout of gene expression leads to strong phenotypes in virus infection assays	Partial knockdown of gene expression may not produce a strong phenotype in virus infection assays
Selection of candidate host factors	Strong phenotypes achieved by gene knockouts result in the identification of candidate genes with higher confidence than those identified through RNAi	Incomplete knockdown and the variability of gene expression combined with off-target effects makes the identification of candidate genes more challenging
Coverage	4–12 sgRNAs per gene	Typically 4–6 shRNAs per gene; up to 30 shRNAs per gene for pooled screening
Off-target effects	Mismatched base pairing may lead to off-target cleavage in the genome (usually in non-coding regions)	Mismatched base pairing may lead to knockdown of off-target mRNAs
Cell types	Wide range of cell types	Wide range of cell types
Analyses	Enrichment of multiple sgRNAs per gene	Enrichment of multiple siRNAs or shRNAs per gene

Figure 24: Comparison of mammalian loss-of-function screening methods (Puschnik et al. 2017)

In a standard pooled CRISPR screen (*Figure 25*), a library of guide RNAs (gRNAs) is introduced into a population of cells, with each individual cell receiving a distinct gRNA that induces a targeted genetic perturbation. Delivery is typically achieved via lentiviral transduction, enabling stable integration of the gRNA cassette into the host genome and thereby allowing perturbations to be traced through the corresponding gRNA sequence. Expression of the CRISPR–Cas nuclease can be established either by stable integration or by transient introduction in the form of a plasmid, viral vector, mRNA, or protein. Following genome editing, the cell population is exposed to a selective pressure, such as drug treatment, viral infection, so that cellular fitness is shaped by the impact of the engineered perturbations. Subsequent quantification of gRNA abundance is performed through high-throughput sequencing, and comparison of gRNA representation across different conditions enables the identification of candidate genes: depletion of gRNAs indicates that disruption of the corresponding gene sensitizes cells to the applied challenge, whereas enrichment suggests a selective advantage conferred by the gene knockout.

Therefore, the experimental design of a typical CRISPR screen consists of four key components: the biological model; the strategy for CRISPR-based perturbation; the selective pressures that shape competition among perturbed cells; and the readout that links the resulting phenotypes to the corresponding gRNAs (Bock et al. 2022).

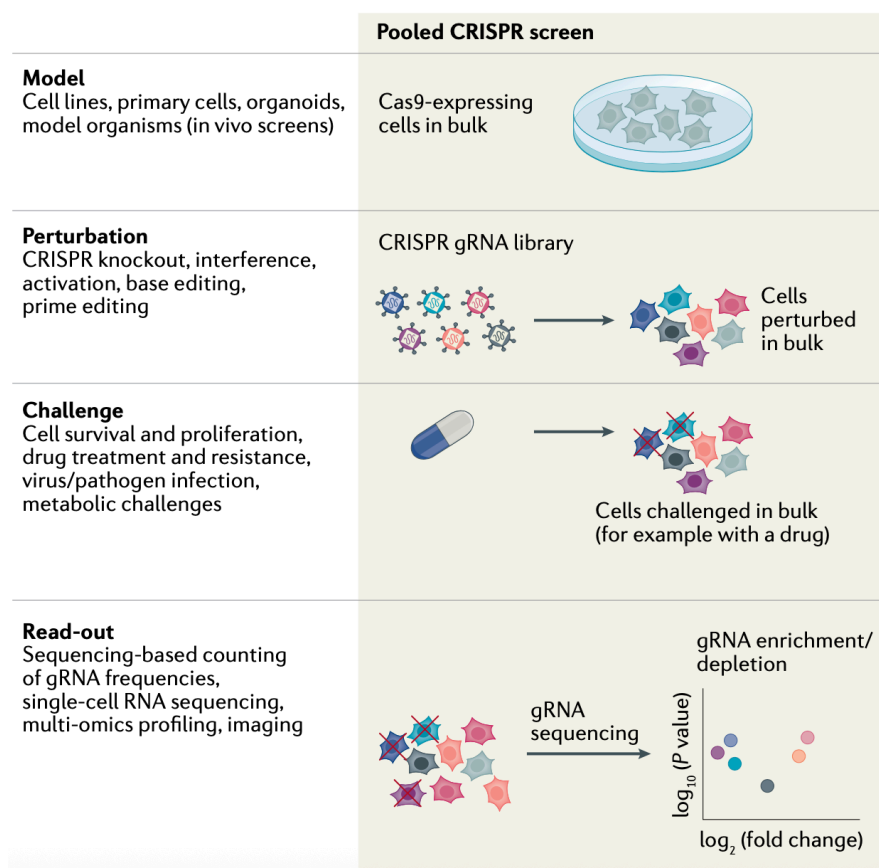


Figure 25: Experimental design for CRISPR screening (Bock et al. 2022)

3- Materials and methods

3.1- Cell line

3.1.1-HepG2 and HEPG2-NCTP

The HepG2 cell line (ATCC® Number: HB-8065™) was isolated from hepatocellular carcinoma and shows an epithelial-like morphology. The HepG2-NTCP cell line was generated from the HepG2 line by lentiviral overexpression of NTCP, which allows HBV infection and viral cycle, and it was used for HBV infection assay.

They were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco – Life Technologies: 4.5 g/L D-glucose, 0.11 g/L Sodium Pyruvate) supplemented with 10% of fetal bovine serum (FBS, Gibco – Life Technologies), 1% of Glutamine (Glu, 100X, Euroclone), 1% of Penicillin-Streptomycin (P/S, 100X, Euroclone), 1% of sodium pyruvate (NaPyr, Gibco – Life Technologies) and 1% of non-essential amino acids (NEAA, Gibco – Life Technologies).

3.1.2- Huh 7-NTCP and Huh 7.5-NTCP

Huh 7 is a differentiated hepatocyte-derived cellular carcinoma cell line. Huh 7.5 cell line was generated from Huh 7 by a missense mutation in the RIG-I gene, which is responsible for type-

1 interferon (IFN-1) response and intracellular sensing of viral genes. Both cell lines were maintained in DMEM, supplemented with 10% FBS, 1% Glu, 1% P/S, 1% NaPyr, and 1% NEAA. Both cell lines were engineered in-house using lentiviral vectors encoding human NTCP, the receptor required for HBV entry.

3.1.3- HEK293TN

HEK293TN is a human embryonic kidney cell line derived from HEK293 (ATCC® number: CRL-1573™) that stably expresses the SV40 large T antigen. This cell line was maintained in DMEM, supplemented with 10% FBS, 1% Glu, 1% P/S, 1% NaPyr, and 1% NEAA.

3.1.4- HepAD38

HepAD38 is a subclone derived from hepatoblastoma cell line HepG2, that expresses HBV genome (genotype D subtype) under the transcriptional control of a tetracycline-responsive promoter in a TET-OFF system: addition of doxycycline suppresses HBV replication, while its removal switches on the process, allowing HBV viral particles to be released in the cell supernatant. HepAD38 were maintained in DMEM/F12 (1:1 mixture of DMEM and Ham's F12) supplemented with 10% FBS, 1% Glu, 1% P/S, 1% NaPyr, and 1% NEAA in the presence of 0.3 µg/ml of doxycycline (tetracycline analogue, SIGMA, D981).

3.1.5- Mouse-passaged Primary Human Hepatocyte (mPHH)

The mPHH used in our experiments were PXB-cells® (PhoenixBio), which were shipped in DMEM without FBS. To ensure optimal culture quality, the medium was promptly replaced upon delivery with hepatocyte growth medium. The recommended medium (dHCGM; J Hepatol. 2006 Apr;44(4):749–57) is commercially available from PhoenixBio and was used to maintain the cultures. Cells were incubated at 37 °C with 5% CO₂, and the medium was refreshed by washing and replacement every 3–4 days. For experiments, 24-well plates were ordered with 4 × 10⁵ hepatocytes per well.

3.2- CellTiter-Blue® Cell Viability Assay

Cell viability was assessed by CellTiter-Blue® Cell Viability Assay (CTB, Promega, G8080).

The system is based on the ability of living cells to convert a redox dye (Resazurin) into a fluorescent end product (Resorufin).

Cells were incubated with the Promega reagent diluted 1:5 in medium for 1h at 37 °C. The fluorescent signal was measured using an Infinite 200 PRO Microplate Reader (TECAN) with a 560Ex/590Em filter set.

3.3- HBV virions production, concentration, and titration

HBV viral stock preparations for infection experiments were produced by maintaining HepAD38 at 90% confluence in doxycycline-free medium. Supernatants were collected twice a week for 1 month, filtered in a 0.45 µm vacuum filtration system, and concentrated 160x through 3-ml cushions of 25% (wt/Vol) sucrose/TNE (40mM Tris-HCl, pH 7,4 – 150 mM NaCl –1mM EDTA) in an SW32 Ti rotor at 22,000 rpm for 16 hours at 4 °C.

At the end of the centrifuge, the obtained supernatant was discarded, and the pellet was resuspended in 200 µl of DMEM plain and left at 4 °C in shaking conditions for at least 4 h to allow the total resuspension of the viral particles. The virus preparation was stored at -80°C.

HBV viral stock was titrated through absolute real-time PCR using HBV-specific primers: HBV viral preparation was lysed to release the genome by the addition of lysis buffer (1mM 1,4-dithiothreitol, 0.2% sodium dodecyl sulfate) and incubated at 95 °C for 10 min. The obtained lysates were then diluted 1:40 in Milli-Q water and used for the Real-Time PCR assay.

The reaction mix (RM) is prepared using the SensiFAST™ SYBR® Lo-ROX Kit master mix (2x, Meridian Bioscience, BIO-94050) and HBV-specific primers: forward (ATTTGTTTCAGTGGTTCGTAGGG) and reverse (CGGTAAAAAGGGACTCAAGATG), both at 5 µM (100 µM stock, desalted, from Eurofins genomics). Real-time PCR steps (*Table 1*) are performed in the Quant Studio (Thermo Fisher Scientific), and data are exported using StepOne Software v2.3.

Real Time PCR Step	Temperature (°C)	Time	Cycle
Initial Denaturation	95	3 min	1
Denaturation	95	5 sec	40
Annealing/Extension	60	20 sec	

Table 1: Real-time PCR steps

The concentration of copies/ml (also indicated as genome equivalent/ml) was obtained by interpolation with a standard curve of known copies/ml of plasmid HBV 1.3-mer WT replicon.

3.4- HBV Infection

Cells were seeded onto multiwell plates previously coated for 1 h with Collagen I, rat tail (Gibco™, A1048301), diluted 1:60 in 20 mM acetic acid, followed by three washes with PBS. After coating, cells were seeded in DMEM and incubated for 24 h at 37 °C with 5% CO₂. Infection was then carried out using the desired multiplicity of genome equivalents (mge). The viral inoculum consisted of DMEM supplemented with 4% PEG8000, 2.5% DMSO, and HBV virions. Cells were exposed to the inoculum for 16 h at 37 °C with 5% CO₂. Following incubation, cells were washed twice with PBS to remove the inoculum and maintained in DMEM containing 2.5% DMSO. The culture medium with 2.5% DMSO was refreshed every 3 days. When Myrcludex B was used as a control, cells were pre-incubated for 1 h at 37 °C with 5% CO₂ with medium containing 200 nM Myrcludex B prior to infection, and the same concentration of Myrcludex B was included in the viral inoculum during the infection step.

3.5- ELISA

3.5.1- ELISA HBeAg

To evaluate the antiviral activity of compounds against HBV, an HBeAg ELISA assay was performed. In this study, we used a sandwich ELISA, in which monoclonal antibodies specific to the HBV protein HBeAg are pre-coated onto the wells of a microplate. HBeAg was chosen as a biomarker because it is a secreted protein that reflects the transcriptional

activity of covalently closed circular DNA (cccDNA), serving as an indirect indicator of viral replication.

Specifically, the HBeAg ELISA kit from Dia.Pro Diagnostic Bioprobes (Italy) was used, following the manufacturer's instructions. The supernatant from HBV-infected cells was directly added to the antibody-coated wells. The plate was incubated for 1 hour at 37 °C to allow HBeAg, if present in the sample, to bind to the immobilised capture antibody.

After the first incubation, the wells were washed five times with the wash buffer provided in the kit to remove unbound materials. During the second incubation step, a detection reagent—consisting of a mixture of two monoclonal anti-HBeAg antibodies conjugated to horseradish peroxidase (HRP)—was added. The plate was incubated for an additional 60 minutes at 37 °C to allow binding of the detection antibodies to the captured antigen.

Following this, the chromogenic substrate tetramethylbenzidine (TMB) was added. TMB reacts with the HRP enzyme to produce a colourimetric signal. After a 20-minute incubation at room temperature, the enzymatic reaction was stopped by adding sulfuric acid (stop solution), resulting in a yellow color.

The absorbance (optical density, OD) was measured at the appropriate wavelength using a plate reader (450nm/620nm). The amount of HBeAg present in the sample was quantified by interpolation from a standard curve generated using the kit-supplied calibrator. All readings were normalized to the mean OD value of untreated, HBV-infected control cells.

Data are expressed as the mean $\pm$ standard deviation of biological triplicates. Results are presented as the percentage of the control (set as 100%).

3.5.2- ELISA HBsAg

To evaluate the antiviral activity of compounds against HBV, an HBsAg ELISA assay was performed. In this study, we used a sandwich ELISA, in which monoclonal antibodies specific to the HBV protein HBsAg are pre-coated onto the wells of a microplate.

Specifically, the HBsAg ELISA kit from Dia.Pro Diagnostic Bioprobes (Italy) was used, following the manufacturer's instructions. The supernatant from HBV-infected cells was directly added to the antibody-coated wells. The plate was incubated for 2 hours at 37 °C to allow HBsAg, if present in the sample, to bind to the immobilised capture antibody.

After the first incubation, the wells were washed five times with the wash buffer provided in the kit (diluted 1:20 in distilled water) to remove unbound materials. During the second

incubation step, a ready-to-use detection mix—containing both a horseradish peroxidase (HRP)-conjugated monoclonal anti-HBsAg antibody and the chromogenic substrate tetramethylbenzidine (TMB)—was added directly to the wells. This single-step incubation allows the detection antibody to bind to the captured antigen while simultaneously initiating the enzymatic reaction. The plate was incubated for 30 minutes at room temperature, during which a colorimetric signal develops as a result of the HRP-TMB reaction. The enzymatic reaction was stopped by adding sulfuric acid (stop solution), resulting in a yellow color. The absorbance (optical density, OD) was measured at the appropriate wavelength using a plate reader (450nm/620nm). The amount of HBsAg present in the sample was quantified by interpolation from a standard curve generated using the kit-supplied calibrator. All readings were normalised to the mean OD value of untreated, HBV-infected control cells. Data are expressed as the mean $\pm$ standard deviation of biological triplicates. Results are presented as the percentage of the control (set as 100%).

3.6- Evaluation of HBV infection and IFN Response in mPHH

3.6.1- Infection time course

mPHHs were infected with 500 mge of HBV, and culture supernatants were collected at different time points post-infection (days 4, 7, 11, and 14). HBV replication was monitored by quantifying HBeAg levels using ELISA.

3.6.2- Evaluation of Sensitivity to IFN

mPHHs were infected with 500 mge of HBV and, starting one day post-infection, treated with either Human Interferon Alpha α 2a (PBL Assay Science™, 11100-1) or Human Interferon α 14 (PBL Assay Science™, 11145-1). Treatments were administered at final concentrations of 500 U/mL and 1000 U/mL, and refreshed every 3 days. Supernatants were collected at days 4, 7, 11, and 14 after infection. HBV replication was assessed by quantifying HBeAg levels using ELISA at each time point, and the signal obtained with IFN treatment was compared with its untreated (UT) condition.

3.7- Assays for the Evaluation of IFN Antiviral Activity in HepG2 Cells

3.7.1- IFN- α 2a Pre-, Post-, and Combined Treatments

HepG2-NTCP cells were seeded 70.000/well in a 48-well plate and subjected to three different treatment strategies with IFN- α 2a (1000 U/mL): (i) pre-treatment (Pre), administered 24 hours after seeding and 24 hours before infection; (ii) post-treatment (Post), administered 16 hours after infection; and (iii) a combined approach (Pre + Post), including both conditions. All infections were performed 48 hours after seeding, and the media with 2,5% DMSO was refreshed 3 days post-infection. Antiviral activity was evaluated 6 days post-infection by HBeAg ELISA, while cell viability was assessed using the CellTiter-Blue (CTB) assay.

3.7.2- Post-infection IFN Treatment Without Refresh in a 6-Day Infection Protocol

HepG2-NTCP B11 cells were seeded 70.000/well in a 48-well plate and infected with HBV 24 hours later at the desired mge. Sixteen hours post-infection, cells were treated with IFNs. Culture medium containing DMSO was refreshed 3 days post-infection. Outcomes were evaluated 6 days post-infection according to the specific experimental readout: (i) percentage of HBcAg-positive cells, representing the proportion of infected cells; (ii) total HBeAg signal, through ELISA; and (iii) cell viability, assessed using the CTB assay. All measurements were compared to the corresponding UT controls.

3.7.3- Post-infection IFN Treatment with Refresh in a 6-Day Infection Protocol

HepG2-NTCP B11 cells were seeded 70.000/well in a 48-well plate and infected with HBV 24 hours later at the desired mge. Infected cells were treated with IFN twice during the course of the experiment: the first treatment was administered 16 hours post-infection, followed by a second treatment 3 days after the initial administration. This protocol consisted of a 6-day infection period, at the end of which outcomes were evaluated by: (i) total HBeAg signal through ELISA, and (ii) cell viability, assessed using the CTB assay. All measurements were compared to the corresponding UT controls.

3.7.4- 12-day HBV infection protocol with IFN refresh

HepG2-NTCP B11 cells were seeded 70,000/well in a 48-well plate and infected with HBV 24 hours later at the desired multiplicity of infection. Sixteen hours post-infection, cells were treated with IFNs. Throughout the experimental setting, IFN treatment was refreshed every 3 days to ensure sustained antiviral pressure. This protocol consisted of a total of 12-day infection period, at the end of which outcomes were evaluated based on two parameters: total HBeAg signal, through ELISA, and cell viability, assessed using the CTB assay. All measurements were compared to the corresponding UT controls.

3.8- 1.1x HBV transfection and IFN treatment in Huh 7-NTCP and Huh 7.5-NTCP cells

3.8.1- Transfection of 1.1x HBV genome into Huh 7-NTCP and Huh7.5-NTCP cells

Huh 7-NTCP and Huh 7.5-NTCP cells were transfected with the HBV full-length 1.1x genome (WT, GeneScript), which is under the control of both endogenous hepatic and CMV promoters. Cells were seeded in 48-well plates at a density of 30,000 cells/well. Twenty-four hours after seeding, transfection was performed using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, L3000015) in Opti-MEM™ medium (Gibco, 31985070), following the manufacturer's instructions. A total of 0.5 µg of HBV 1.1x plasmid DNA was used per well. Six hours post-transfection, the medium was replaced with fresh complete culture medium.

3.8.2- Antiviral evaluation of IFN treatments in 1.1x HBV-transfected Huh 7-NTCP cells

1.1x HBV transfection was performed in Huh 7-NTCP cells as described above. Six hours post-transfection, the medium was replaced, and cells were treated with a single dose (500 U/mL) of IFN-α2a, IFN-α14, IFN-λ1, or IFN-λ2. An additional IFN treatment refresh was performed 3 days post-transfection. Outcomes were assessed at day 6 post-transfection. Viral antigen levels were quantified by HBeAg ELISA, while cell viability was determined using the CTB assay. All results were normalized to the corresponding UT controls.

3.9- RNA Launch

3.9.1- RNA *in vitro* transcription

For *in vitro* transcription, the plasmids pGEM-3Z-T7 HBV genotype A (GenBank Accession No. MN172185) and pCMV T7 eGFP (BPK1098, Addgene Plasmid #133962) were used. Each plasmid required linearization with a specific restriction enzyme before transcription with T7 RNA polymerase. 10 µg of pGEM-3Z-T7 HBV genotype A were linearized with NotI-HF® (NEB® R3189L), and 10 µg of pCMV T7 eGFP were linearized with PmeI® (NEB® R0560L). Digestions were carried out in a total reaction volume of 100 µL under the recommended conditions at 37 °C for 4 hours, followed by enzyme inactivation at 65 °C for 20 minutes. DNA was purified by sodium acetate precipitation: to each 100 µL reaction, 10 µL of 3 M sodium acetate, 250 µL of 100% cold ethanol, and 1 µL of glycogen were added. Samples were incubated at –80 °C for 30 minutes and centrifuged at 18,000 g for 30 minutes at 4 °C. Pellets were washed with 250 µL of 70% cold ethanol, centrifuged again at 18,000 g for 20 minutes at 4 °C, and finally resuspended in 50 µL of nuclease-free water.

In vitro transcription was carried out using the RiboMAX™ Large Scale RNA Production System (Promega, P1300) according to the manufacturer's instructions. Reactions were incubated at 37 °C for 2–4 hours with T7 RNA polymerase, followed by DNase treatment using the same kit. RNA was purified by LiCl precipitation as described in the HiScribe® T7 ARCA mRNA kit (NEB, E2065S) manual. Following RNA isolation and purification, capping and polyadenylation were performed using the T7 mScript Standard mRNA Production System (CELLSCRIPT, C-MS100625) according to the Cap 1 mRNA protocol provided by the manufacturer. The resulting RNA was again purified by LiCl precipitation, and then RNA was quantified by nanodrop.

3.9.2- Post-RNA Launch Interferon Treatment on HBV Replication

Huh 7.5-NTCP cells were seeded at a density of 75,000 cells per well in 24-well plates. After 24 hours, the culture medium was replaced with DMEM supplemented with 2% FBS, 1% GlutaMAX™, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids (NEAA), and HBV GtA RNA was transfected at the desired amount using Lipofectamine™ 2000 Transfection Reagent (Invitrogen™ 11668019), following the manufacturer's instructions. Six hours post-transfection, the medium was replaced with DMEM containing 10% FBS, 1% GlutaMAX™, 1% penicillin-streptomycin, 1% sodium

pyruvate, 1% NEAA, and 2% DMSO. Interferon treatment was then applied in this medium at the desired concentrations. Three days post-transfection, interferon treatment was refreshed, maintaining the cells in 2% DMSO. HBsAg levels were quantified six days post-transfection to assess the effect of interferon on viral replication using an HBsAg ELISA, and signals were compared to UT controls without interferon.

3.9.3- Pre-RNA Launch Interferon Treatment on HBV Replication

Huh 7.5-NTCP cells were seeded at a density of 60,000 cells per well in 24-well plates. IFN exposure was initiated 24 hours after cell seeding by exchanging the culture medium with fresh medium containing the appropriate concentration of IFN. 24 hours post IFN-treatment, culture medium was replaced with DMEM supplemented with 2% FBS, 1% GlutaMAX™, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% non-essential amino acids (NEAA), and HBV GtA RNA was transfected at the desired amount using Lipofectamine™ 2000 Transfection Reagent (Invitrogen™ 11668019), following the manufacturer's instructions. Six hours post-transfection, the medium was replaced with DMEM containing 10% FBS, 1% GlutaMAX™, 1% penicillin-streptomycin, 1% sodium pyruvate, 1% NEAA, and 2% DMSO. After three days, the medium was refreshed, maintaining the cells in 2% DMSO. HBsAg levels were quantified six days post-transfection to assess the effect of interferon on viral replication using an HBsAg ELISA, and signals were compared to UT controls without interferon.

3.9.4- eGFP RNA Launch with Pre- and Post-interferon Treatment

Experiments assessing the effect of interferon on GFP RNA launch were performed in parallel to those described above for HBV RNA. Huh 7.5-NTCP cells were seeded at a density of 60,000 cells per well in 24-well plates and exposed to IFN either 24 hours prior to RNA transfection (pre-treatment) or following RNA transfection (post-treatment), as previously detailed. eGFP RNA was transfected using 1µg/well using Lipofectamine™ 2000 Transfection Reagent (Invitrogen™ 11668019), following the manufacturer's instructions. Culture conditions, IFN concentrations, and treatment timelines were identical to those described for HBV RNA experiments, except that no DMSO was added after transfection. Transfection efficiency and interferon effects were evaluated three days post-transfection by

flow cytometry, quantifying the percentage of GFP-positive cells and comparing IFN-treated samples with UT controls.

3.10- Cell Seeding in DMSO and Evaluation of IFN Treatment in HBV-Infected HepG2-NTCP B11 Cells

HepG2-NTCP B11 cells were seeded at a density of 80,000 cells per well in 48-well plates in the presence of 2.5% DMSO. Twenty-four hours post-seeding, cells were infected with HBV at the desired mge. Infected cells were treated with IFN twice during the course of the experiment: the first treatment was administered 16 hours post-infection, followed by a second treatment 3 days after the initial administration. Experimental outcomes were assessed 6 days post-infection by measuring secreted HBeAg levels in culture supernatants using ELISA, and by evaluating cell viability through the CTB assay. All experimental conditions were compared with the corresponding UT control samples.

3.11- Validation of HBV GFP Reporter System

3.11.1- HBV 1.1X Transfection and Quantification by Real-Time PCR

Cells were transfected with the full-length HBV 1.1× genome (wild type, GeneScript), which is driven by both endogenous hepatic and CMV promoters. HepG2-NTCP B11 cells were seeded in 24-well plates at a density of 100,000 cells per well. Twenty-four hours after seeding, transfection was performed using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, 11668019) in Opti-MEM™ medium (Gibco, 31985070), following the manufacturer's instructions. For each well, 0.5 µg of HBV 1.1× plasmid DNA (wild type or reporter variants) was used. Six hours post-transfection, the medium was replaced with fresh complete culture medium. Where indicated, tenofovir was added at a final concentration of 0.1 µM to inhibit HBV reverse transcription and subsequent viral replication.

Supernatants were collected six days after transfection, and HBV DNA levels were quantified by real-time PCR. Before analysis, supernatants were treated with DNase I (1 U/well, Sigma-Aldrich™, AMPD1) according to the manufacturer's protocol. Following DNase treatment, samples were lysed to release viral genomes by adding lysis buffer (1 mM 1,4-dithiothreitol, 0.2% sodium dodecyl sulfate) and incubating at 95 °C for 10 min. Four microliters of a 1:40 dilution in MQ water were used as a template for PCR.

Reactions were prepared using the SensiFAST™ SYBR® Lo-ROX Kit (2×, Meridian Bioscience, BIO-94050) with specific primers (5 µM final concentration; 100 µM stock, desalted; Eurofins Genomics). HBV-specific primers were: forward 5'-ATTTGTTCACTGGTTCGTAGGG-3' and reverse 5'-CGGTAAAAAGGGACTCAAGATG-3'. Ampicillin-specific primers were: forward 5'-TGCTTAATCAGTGAGGCACCTA-3' and reverse 5'-AGCCCTCCCGTATCGTAGTTAT-3'. PCR cycling conditions and instrumentation are described in Section 3.3.

For each technical duplicate, the mean Ct value was calculated. ΔC_t values were determined as the difference between the sample mean Ct and the control mean Ct. Relative Quantification (RQ) was calculated as $2^{-\Delta C_t}$, representing the relative abundance of HBV genomes compared to the wild-type 1.1× construct.

3.11.2- Split-GFP Complementation Assay in HEK293TN

For transfection experiments in HEK293TN cells, 80,000 cells were seeded per well in 24-well plates. The split-GFP system was employed using GFP1-10 (Addgene #70219) in combination with either the wild-type GFP11 construct fused to the light clathrin chain (Addgene #70217) or a modified version containing HBeAg fused to GFP11. Plasmids encoding GFP11 constructs and GFP1-10 were co-transfected at a 1:3 ratio (500 ng GFP11 and 1500 ng GFP1-10 plasmid) using Lipofectamine™ 2000 Transfection Reagent (Invitrogen™, 11668019), according to the manufacturer's instructions. Six hours post-transfection, the medium was replaced with fresh culture medium. Cells were analyzed three days later by flow cytometry to quantify the percentage of GFP-positive cells, thereby assessing GFP signal reconstitution through the split-GFP system.

3.11.3- Western blot on HBeAg-GFP11 constructs

HEK293TN cells were seeded at a density of 300,000 cells per well in 6-well plates and transfected with Lipofectamine™ 2000 Transfection Reagent (Invitrogen™, 11668019), following the manufacturer's instructions. Each well was transfected with 3 µg of plasmid DNA encoding one of the six HBeAg-GFP11 variants (V1-V6), HBV 1.3X (Addgene

plasmid #65459), p17, p22, or p25 (kindly provided by the Jianming Hu laboratory). Six hours after transfection, the culture medium was replaced with fresh medium.

Cells were harvested three days post-transfection and lysed in RIPA buffer supplemented with complete protease inhibitor cocktail (Roche), sodium fluoride (10 mM), and sodium orthovanadate (1 mM) (both from Sigma). Lysates were resolved on 4–12% SDS-PAGE gels (BOLT bis-tris precast gel, ThermoFisher) at a constant voltage of 200 V for ~45 minutes, and subsequently transferred to 0.2 µm nitrocellulose membranes (Amersham™ Protran®, GE Healthcare) at 90 V for 1 hour at 4 °C.

Membranes were blocked in 5% milk prepared in TBS with 0.1% Tween-20 (TBS-T) for 1 hour with gentle rocking, washed three times with TBS-T, and incubated overnight at 4 °C with primary antibodies diluted 1:1000 in 5% BSA/TBS-T. After washing, membranes were incubated with the appropriate secondary antibody (1:5000 dilution in 5% milk/TBS-T), washed three times, and developed using ECL detection reagent (Amersham™, GE Healthcare). Chemiluminescent signals were acquired with an iBright FL 1500 imaging system (ThermoFisher).

The following primary antibodies were used: anti-GFP11 (Biorbyt, orb621407) and anti-HBV core polyclonal antibody (Sigma Aldrich Cell Marque, 216A-14-RUO).

3.11.4- GFP1-10 Engineering

To generate endoplasmic reticulum (ER)-targeted GFP1-10 constructs, the plasmid pcDNA3.1-GFP1-10 (Addgene #70219) was engineered by molecular cloning. Specifically, a signal peptide derived from binding protein BiP (sequence: ATGAAGCTCTCCCTGGTGGCCGCGATGCTGCTGCTGCTCAGCGCGGCGCGGGC C) was inserted at the N-terminal region, upstream of the GFP1-10 start codon, to direct protein localization to the ER. At the C-terminal end of GFP1-10, a KDEL motif (Lys-Asp-Glu-Leu) was introduced as an ER retention signal, followed by a stop codon to terminate translation.

This strategy resulted in the generation of two distinct constructs:

1. pcDNA3.1-GFP1-10 SP+KDEL, containing both the BiP-derived signal peptide at the N-terminus and the KDEL ER-retention motif at the C-terminus.
2. pcDNA3.1-GFP1-10 SP, containing only the BiP signal peptide at the N-terminus.

3.11.5- Generation of Stable NTCP Cell Lines Expressing GFP1-10

SP+KDEL

Lentiviral vectors encoding GFP1-10 SP+KDEL were produced using a standard calcium phosphate co-transfection procedure with third-generation helper and transfer plasmids. The following helper plasmids (all from Addgene, provided by the Didier Trono laboratory) were used: pMD2.G/VSV-G (#12259), pRSV-Rev (#12253), and pMDLg/pRRE (#12251). The transfer vector pLENTI_GFP1-10 SP+KDEL was obtained by cloning pcDNA3.1-GFP1-10 SP+KDEL into the backbone pLenti-CMV-GFP-Hygro (Addgene #17446, from Eric Campeau & Paul Kaufman).

HEK293Tn cells were seeded at 5×10^6 cells per 15-cm dish in complete DMEM. The following day, two hours before transfection, the medium was replaced with 22.5 ml of Iscove's Modified Dulbecco Medium (IMDM, Gibco) supplemented with 10% FBS (Gibco), 1% glutamine, 1% penicillin-streptomycin (Euroclone, 100X), 1% sodium pyruvate (Gibco), and 1% non-essential amino acids (Gibco).

Co-transfection was performed with the following plasmids: 40 μ g of pLenti-GFP1-10 SP+KDEL, 12.5 μ g of pMDLg/pRRE, 6.25 μ g of pRSV-Rev, and 9 μ g of pcDNA3.1-VSV-G. Plasmids were mixed in 1.125 ml of Milli-Q water, followed by the addition of 156 μ l of CaCl_2 . After 5 minutes at room temperature, 1.25 ml of HBS was added, and the mixture was incubated for 15 minutes before being added dropwise to the cells. Transfected cells were incubated at 37 °C for 12–14 hours.

The following day, the medium was replaced with 16 ml of fresh IMDM per dish. At 30 hours post-transfection, viral supernatants were collected, clarified through a 0.45 μ m filter, and concentrated by ultracentrifugation at 20,000 rpm for 2 hours at 4 °C. Viral pellets were resuspended in 80 μ l of PBS, aliquoted, and stored at –80 °C.

Huh 7-NTCP, Huh 7.5-NTCP, and HepG2-NTCP cells were transduced with the GFP1-10 SP+KDEL lentiviral vector. Forty-eight hours post-transduction, stable clones were selected by puromycin (Gibco™, A1113803) at 4 μ g/ml for Huh 7-NTCP and Huh 7.5-NTCP, and at 2 μ g/ml for HepG2-NTCP. Selected cell lines were maintained in their respective standard culture media supplemented with puromycin at the indicated concentrations.

3.11.6- Complementation Assay in Stable GFP1-10 SP+KDEL Cell Lines

To assess split-GFP complementation, stable Huh 7-NTCP GFP1-10 SP+KDEL and Huh 7.5-NTCP GFP1-10 SP+KDEL cells were seeded at 40,000 cells per well in 24-well plates, while HepG2-NTCP GFP1-10 SP+KDEL cells were seeded at 100,000 cells per well. Transfections were performed 24 hours post-seeding with Lipofectamine™ 2000 Transfection Reagent (Invitrogen™, 11668019), according to the manufacturer's instructions. Each well received 2 µg of plasmid DNA encoding one of the six HBeAg-GFP11 variants (V1–V6). Three days post-transfection, cells were analyzed by flow cytometry to quantify the percentage of GFP-positive cells, thereby evaluating GFP signal reconstitution through the split-GFP complementation system.

3.12- Intracellular Staining of HBcAg in HBV-Infected Cells

To assess HBV infection, intracellular staining of the core antigen (HBcAg) was performed. Infected cells were collected, washed in PBS, and fixed/permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences, 554714) according to the manufacturer's protocol. After permeabilization, cells were blocked with 0.5% normal goat serum (NGS) and subsequently incubated with an anti-HBV core polyclonal antibody (Sigma Aldrich Cell Marque, 216A-14-RUO) at a 1:500 dilution. Following washes with BD Perm/wash™, cells were stained with a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 647 (1:200 dilution) for 30 minutes at room temperature. After final washes, cells were fixed in 1% paraformaldehyde. Samples were then analyzed by flow cytometry, and the percentage of Alexa Fluor 647–positive cells was quantified as a measure of HBV infection.

4- Results

4.1- Identification of an *In Vitro* HBV Infection Model Sensitive to Interferon

The first part of the project was focused on the identification of an *in vitro* model sensitive to IFN antiviral action on HBV infection, which enables to perform a positive CRISPR-Cas9 screening to uncover the cellular determinants involved in the antiviral action of interferon against HBV. *In vitro* models provide an excellent approach to study HBV infection that does not require *in vivo* use of animals or patient-derived material. Different cell lines served as models for HBV infection, which have their individual advantages and limitations. The main cell lines used for HBV research are hepatoma-derived cell lines, such as HepG2 or Huh 7 derivatives, in which expression of the HBV receptor (NTCP) is obtained ectopically, and primary human hepatocytes (PHHs).

Thus, HepG2, Huh 7, and Huh 7.5 cells, all derived from human hepatocellular carcinoma, have been genetically modified to express the sodium-taurocholate co-transporting polypeptide (NTCP) receptor, rendering them susceptible to HBV infection. Despite the expression of this key receptor, these cell lines exhibit some limitations, including the requirement of high viral titer to achieve efficient infection and the absence of viral spread. Conversely, primary human hepatocytes are considered to be a more physiologically relevant *in vitro* model as they more closely mimic the *in vivo* hepatic environment. However, there are limitations to be considered, such as high cost, limited availability, and donor-to-donor variability.

Type I IFN-sensitive cell lines may represent useful tools to explore IFN pathways and to suggest potential effectors involved in the cellular antiviral response. However, their application is complicated by the wide variability in IFN responsiveness across cell lines and genetic backgrounds. The key challenge we faced, thus, was to identify an *in vitro* model system combining two critical features: high susceptibility to HBV infection and intact sensitivity to IFN, ensuring that viral replication can be efficiently suppressed upon treatment. Such a model is essential for the success of a genetic screen, which requires both a high percentage of HBV-infected cells and a robust antiviral response to IFN. This ensures that, after performing a CRISPR-Cas9 knockout, using a pooled library targeting a set of

Interferon Stimulating Gene (ISG), and subsequently treating with IFN, only cells lacking critical IFN effectors remain HBV-positive. These cells can be selected as hits, thereby revealing critical effectors of the antiviral IFN effect. To achieve this, a reliable method to identify and sort HBV-infected cells is also required.

Finally, it is essential to establish a model in which IFN remains effective in suppressing viral replication and production even after infection establishment (i.e., *post-infection*), as it more accurately reflects the clinical scenario in which patients are treated once infection has already occurred.

Based on these assumptions, we started to characterize several *in vitro* HBV infection systems, focusing particularly on infection efficiency and responsiveness to selected type I IFNs.

4.1.1-Mouse-passaged Primary Human Hepatocytes are susceptible to IFN treatment *in vitro*

As a first *in vitro* model to evaluate, we considered primary human hepatocytes, which are a model that most closely recapitulates hepatic functions. Considering that clinical evidence supports the efficacy of IFN in patients, its activity is expected to be confirmed also in this biologically relevant model. In particular, we decided to use mouse-passaged Primary Human Hepatocyte (mPHH), which are specialized human hepatocyte cell lines obtained from chimeric humanized liver mice. These mice are generated by transplanting human hepatocytes into immunodeficient mice, such as uPA/SCID or TK-NOG models, in which the endogenous mouse hepatocytes are selectively depleted. Over time, the human hepatocytes engraft, expand, and repopulate the liver, leading to a chimeric organ composed predominantly of functional human liver cells. Hepatocytes harvested from these humanized mice retain key characteristics of primary human hepatocytes, including liver-specific gene expression, metabolic activity, and responsiveness to cytokines such as interferons (PhoenixBio, www.phoenixbio.com).

In order to optimally assess HBV replication in mPHH cells *in vitro*, we performed a time-course analysis. HBV replication was quantified based on secreted HBeAg levels at different timepoints. As shown in *Figure 26*, HBeAg levels progressively increased from day 4 to day 11 post-infection, peaking at day 11 and slightly decreasing till day 14.

mPHH cells demonstrated a substantially higher replication efficiency compared to the maximal levels typically observed in HepG2-NTCP cells infected under the same conditions.

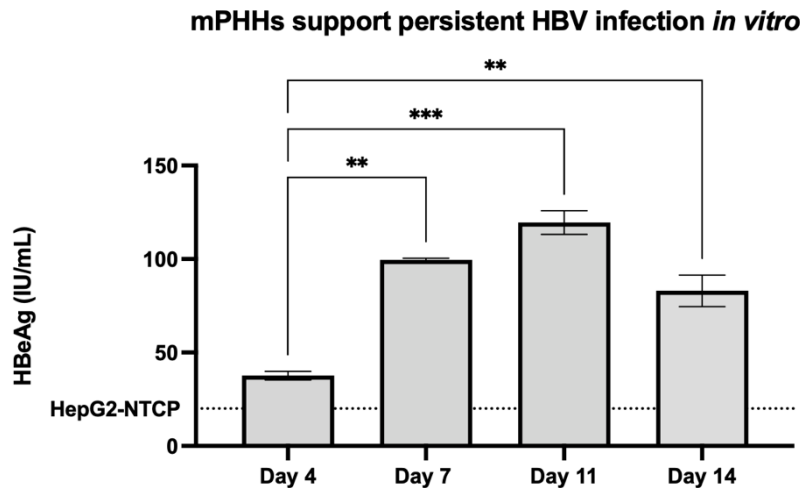


Figure 26: Infection time course of mPHH. mPHH were infected using 500 mge of HBV, and at different time points after infection (Day 4, 7, 11, and 14), we assessed the replication signal through ELISA HBeAg. The signal obtained from HepG2 cells under the same infection conditions is represented by a dashed line. Data are presented as mean $\pm$ standard deviation (SD). Brown-Forsythe test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

These findings conclusively demonstrate that mPHH cells efficiently support HBV infection, as evidenced by the sustained and high-level secretion of HBeAg.

Thereafter, we evaluated the effect of interferon treatment on mPHH-infected cells. Firstly, we tested IFN- α 2a, as it represents the most commonly used IFN subtype in clinical and therapeutic settings. Given its well-characterized profile and proven efficacy in patients, it is expected to be effective also in the mPHH cellular model. Moreover, recent discoveries show that among all IFN- α subtypes tested, IFN- α 14 demonstrated the highest antiviral potency against HBV *in vitro*. Indeed, IFN- α 14 was shown to be 100-fold more effective than the widely used IFN- α 2a in suppressing HBV cccDNA transcription and viral antigen production, making it the most suitable subtype for *in vitro* experiments on HBV infection (Chen et al. 2021). Given the evidence supporting its superior antiviral activity, we decided to include both IFN- α 2a and IFN- α 14 in our study.

To evaluate the antiviral efficacy of IFNs, we administered the treatment post-infection to mimic the therapeutic setting. A time-course experiment was performed in HBV-infected

mPHHs, quantifying HBeAg levels in the culture supernatant at various time points post-infection. HBeAg was used as a surrogate marker of HBV replication, and it was compared to the untreated condition (UT), which corresponds to HBV infection in the absence of IFN treatment. As shown in *Figure 27*, treatment with IFN- α 2a resulted in a dose-dependent suppression of HBeAg secretion, and at all examined time points, the decrease was markedly significant. In particular, the most pronounced reduction was observed by day 14 using 1000U/mL, with a reduction of 84% of the signal. These results collectively indicate that the antiviral activity of IFN- α 2a is recapitulated in this *in vitro* model.

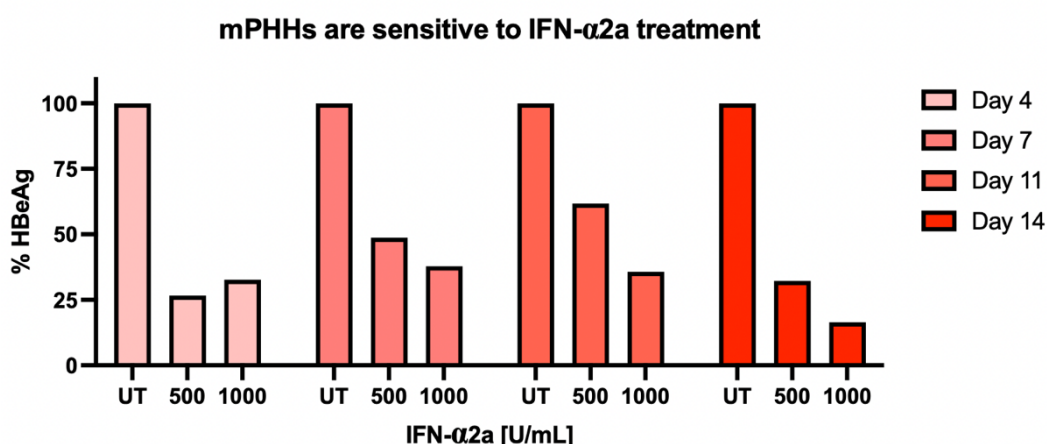


Figure 27: IFN- α 2a treatment using mPHH. Cells were infected with 500mge of HBV, and one day post-infection, cells were treated with both concentrations of 500 U/mL and 1000 U/mL and compared to the untreated control. IFN treatment was refreshed every 3 days.

Figure 28 depicts the results of the same experiment performed with IFN- α 14 treatment, which led to a marked suppression of HBeAg secretion at both tested concentrations. A pronounced reduction was already evident by day 4, nearly abolishing the detectable signal. This effect persisted throughout the experiment, with HBeAg levels consistently maintained at or below 5% across all subsequent time points.

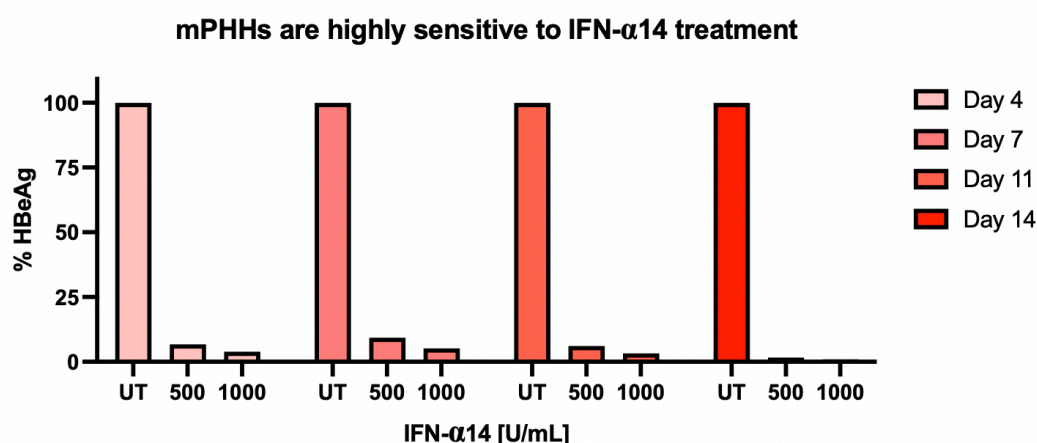


Figure 28: IFN- α 14 treatment using mPHH. Cells were infected with 500mge of HBV, and one day post-infection, cells were treated with both concentrations of 500 U/mL and 1000 U/mL and compared to the untreated control. IFN treatment was refreshed every 3 days.

Therefore, IFN- α 14 elicited a more pronounced reduction in HBeAg levels compared with IFN- α 2a. These findings indicate that IFN- α 14 exerts greater efficacy than IFN- α 2a in suppressing HBV replication in primary human hepatocytes, even at equivalent doses. Overall, our results demonstrate that mPHH cells exhibit a strong response to interferon treatment, even when used after infection. However, their application for a CRISPR-Cas9 screening is limited by several significant drawbacks: they are costly and technically challenging to handle, which could limit the ability to obtain the large number of cells required for the screening. Moreover, their low detachment efficiency represents a major limitation, as detaching mPHH cells from culture surfaces leads to significant cell loss. This issue is particularly critical in the context of CRISPR/Cas9 screening, where efficient recovery of viable cells is essential for the accurate selection of positively responding populations by flow cytometry. Excessive cell loss during detachment could result in the accidental elimination of true positive hits, thereby compromising the reliability and overall success of the screening. In conclusion, even though mPHH cells have high biological relevance in HBV infection models, these issues make them unsuitable for CRISPR-Cas9 screening.

4.1.2- Limited Antiviral Efficacy of IFNs on HBV Infection in HepG2-NTCP Cells

4.1.2.1- Assessing the Antiviral Activity of IFN- α in HepG2 Cells Across Distinct Treatment Conditions

Given the limitations of primary cells in our study, we considered the use of immortalized cell lines infected with HBV. Among the most commonly used *in vitro* models for HBV studies, we selected the HepG2 cell line. HepG2 is a human hepatocellular carcinoma cell line derived from the liver tissue of a well-differentiated hepatocellular carcinoma. These cells retain many hepatic features, including the expression of liver-specific proteins and enzymes, and are widely used due to their stable growth, ease of manipulation, and capacity to support HBV replication. Specifically, for our experiments we exploited a monoclonal HepG2 cell line engineered in-house to express NTCP. The NTCP receptor was introduced via lentiviral transduction, followed by single-cell cloning. Among the isolated clones, one called B11 was selected based on its higher susceptibility to infection.

As previously tested on mPHHs, we firstly evaluated the sensitivity of HepG2 NTCP B11 cells to IFN- α 2a. *Figure 29* illustrates the effect of different IFN- α 2a treatment conditions on viral infection and cell viability. Cells were treated at distinct time points: before infection (Pre), following infection (Post), or both before and following infection (Pre + Post).

Both the Pre + Post and Pre interferon treatments exhibited similar levels of antiviral potency, but resulted in increased cell toxicity. Post-treatment condition, the most relevant to our experimental goals, maintained high cell viability but exhibited only moderate antiviral efficacy.

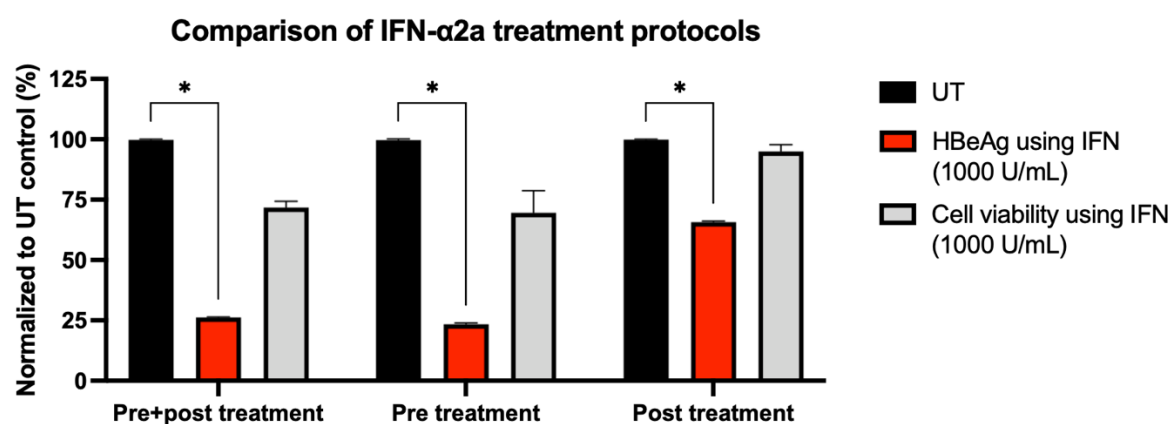


Figure 29: IFN-α2a treatment using HepG2 NTCP. Cells were infected using 500 mge and exposed to IFN using three distinct treatment strategies: pre-treatment (Pre) 24 hours before infection; post-treatment (Post) 16 hours after infection; a combined approach (Pre + Post) including both. IFN-α2a was used at a single concentration of 1000U/mL among all the conditions. Results were assessed 6 days post-infection by HBeAg ELISA. Data are presented as mean ± SD. Tukey's multiple comparisons test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

In conclusion, conditions associated with a marked reduction in viral replication were accompanied by high cytotoxicity, whereas less toxic conditions exhibited only limited antiviral efficacy. These challenges limit the applicability of this experimental layout to our screening.

4.1.2.2- Evaluating the Impact of HBV Infection Intensity on IFN-α14 Antiviral Activity

A high percentage of infected cells is essential for CRISPR-Cas9 screening, as only virus-positive cells are included in the final analysis. When infection rates are low, much larger cell numbers are required to achieve sufficient representation. In contrast, starting the screening with higher infection levels makes the procedure more manageable, provided that the IFN-induced antiviral response still limits viral replication effectively. For this aim, we assessed the cellular infection rate employing different HBV concentrations. Based on prior experiments, infection with 500 mge results in approximately 30% of infected cells. To enhance the infection rate, we tested higher mge doses, specifically 1000, 5000, and 10000.

As shown in *Figure 30*, Infection with 1,000 MGE resulted in approximately 65% of cells being infected. At higher doses, such as 5,000 and 10,000 MGE, the increase in infection rate was less significant, reaching a plateau at around 80–85%. This suggests that the system was approaching its maximal infectivity.

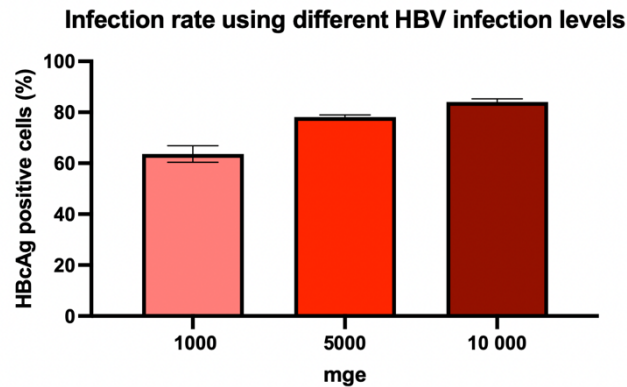


Figure 30: Infection rate using 1000, 5000, and 10000 mge. 6 days after infection, HepG2-NTCP B11 were stained using an anti-HBcAg antibody in order to assess the HBV-positive cells through flow cytometry. Data are presented as mean $\pm$ SD. Differences were not statistically significant.

Given these substantially higher levels of infection achieved with increased mge doses, we decided to employ these infection conditions in subsequent experiments to assess the IFN response. *Figure 31* illustrates the antiviral effect of IFN- α 14 on HBV infection at the three previously tested infection doses: 1000, 5000, and 10,000 mge. The analysis evaluates three key parameters: the proportion of effectively infected cells positive to HBcAg, the overall viral antigen load through HBeAg, and cell viability. Treatment with IFN- α 14 resulted in a modest reduction in both the percentage of HBcAg-positive cells and the overall HBeAg signal across all mge conditions. Cell viability remained unaffected by IFN- α 14, confirming that the observed antiviral effect was not related to cellular cytotoxicity but to the actual viral replication reduction. As expected, the most pronounced reduction was observed at the lowest infection dose used. Nevertheless, residual HBV levels remained elevated, with the maximum reduction reaching only 75% of the original signal, insufficient to meet the objectives of our experimental design.

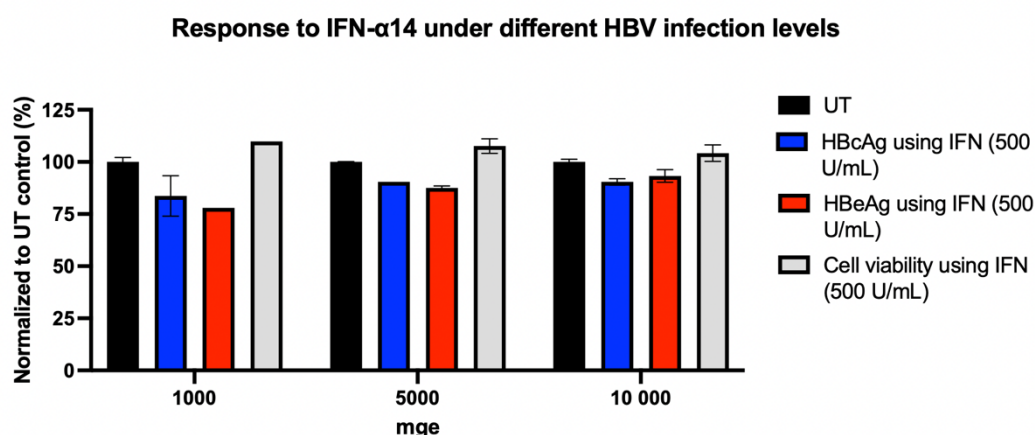


Figure 31: IFN- α 14 effect using different rates of infection. IFN was used at a single dose of 500U/mL 16 hours after HBV infection, and the results were assessed 6 days post-infection. The analysis evaluates three key parameters: (1) the percentage of HBV core antigen (HBcAg)-positive cells, representing the proportion of effectively infected cells; (2) the total HBeAg signal, reflecting the overall viral antigen load regardless of cell count; and (3) cell viability, assessed by Cell Titer-Blue (CTB) assay. Each of the three parameters was compared to the relative untreated (UT) condition. Data are presented as mean $\pm$ SD. Differences were not statistically significant.

4.1.2.3- Dissecting the Antiviral Potential of IFN- α 14 in HBV-Infected HepG2 Cells

Following the results obtained, we decided to decrease the mge to 500 and to focus exclusively on IFN- α 14 in the post-treatment condition. This choice was driven by the observation that IFN- α 14 displayed superior efficacy compared to IFN- α 2a in mPHHs. Moreover, we maintained only the post-treatment setting, as this approach allows us to concentrate on an experimental model that better reflects a curative rather than a prophylactic treatment scenario. *Figure 32* shows the dose-response relationship between increasing concentrations of IFN- α 14 and the expression of HBeAg.

A clear dose-dependent decrease in HBeAg levels is observed, indicating the antiviral effect of interferon. Cell viability it's not affected by IFN treatment, we can conclude that the antiviral effect shown is only due to the drug effect. However, a residual signal of approximately 25% remains, which could be a huge background noise considering the screening.

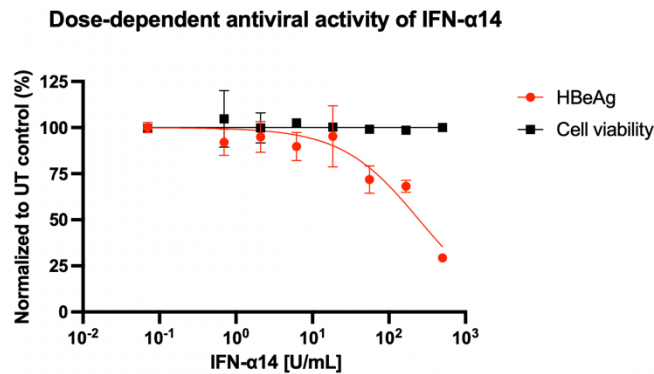


Figure 32: Curve dose-response using IFN- α 14. HepG2-NTCP B11 were infected using 500 mge and treated 16 hours post-infection with different doses of IFN. Results were assessed 6 days after infection by HBeAg ELISA and compared to the cell viability. Data are presented as mean $\pm$ SD.

Following these observations, we decided to modify the experimental protocol. In previous experiments, the viral signal was assessed 6 days post-infection after a single interferon treatment. In the new protocol, the analysis is conducted 12 days post-infection, with interferon treatment being refreshed every 3 days. Given the implementation of a revised protocol with the aim of achieving improved optimization, we reconsidered the use of higher infection doses to determine whether the new conditions would remain effective under increased viral load.

Figure 33 illustrates the effects of IFN- α 14 treatment on HBV-infected cells under the new experimental conditions. The results show that cell viability is stable across the different infection doses, with only a slight decrease observed at higher mge levels, suggesting that IFN- α treatment does not significantly compromise cell viability. HBeAg levels were reduced in all IFN-treated groups compared to untreated controls, even at the highest viral load.

Prolonged IFN- α 14 exposure across different HBV infection levels

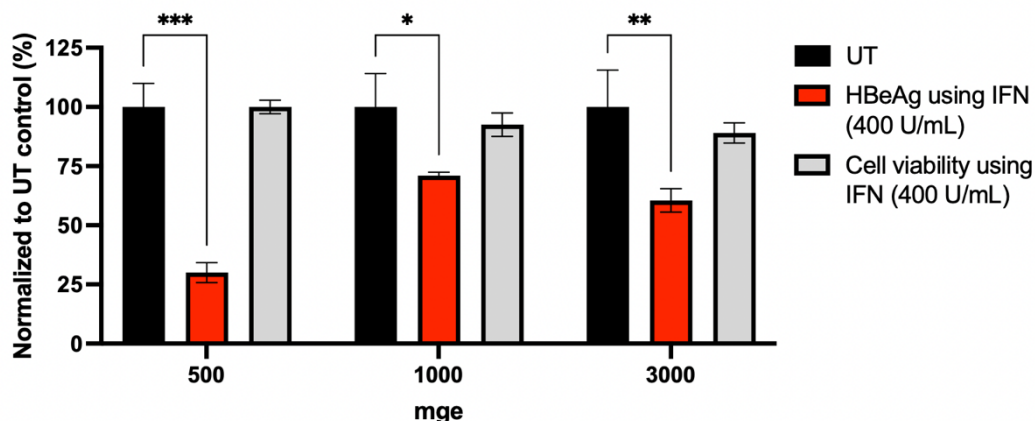


Figure 33: New protocol using IFN- α 14 with different HBV concentrations (500, 1000, and 3000). HepG2-NTCP B11 were infected with HBV and 16 hours later treated with IFN (400U/mL). IFN treatment was refreshed every 3 days, and the results were assessed by HBeAg ELISA 12 days post-infection. Data are presented as mean $\pm$ SD. Data are presented as mean $\pm$ SD. Tukey's multiple comparisons test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

The implementation of the novel experimental protocol resulted in a significant reduction in viral replication, achieving up to 75% decrease in HBeAg levels. Moreover, a greater reduction in viral signal was observed even at higher levels of infection compared to previous experimental conditions.

These latest results suggest that the new protocol may be more effective at higher viral loads, improving the overall condition for our screening. However, it is important to note that, despite the overall efficacy, the viral signal is still not decreasing beyond ~25%, indicating a potential limit of IFN responsiveness in this system.

4.1.2.4- Evaluating the Efficacy of Type I and Type III Interferons in HBV-Infected HepG2 Cells

Therefore, as a next step, we examined other interferons that are not members of the IFN- α superfamily, focusing on type III interferons known as IFN- λ , and specifically two of the four known subtypes: IFN- λ 1 and IFN- λ 2. In *Figure 34* is displayed the evaluation of the antiviral activity of these two IFNs versus HBV infection. As previously described, treatment efficacy was assessed through HBeAg expression, and cell viability was measured to evaluate the potential cytotoxic effects of the treatments. IFN- λ 1 showed a pronounced antiviral effect, even if it affected cell viability. On the other hand, IFN- λ 2 induced a lower viral replication reduction compared to IFN- λ 1 while maintaining a higher level of cell

viability. These results highlight the need to balance antiviral efficacy and cellular fitness when designing screening conditions, since reductions in viral markers may partly reflect IFN-induced cytotoxicity rather than true antiviral activity.

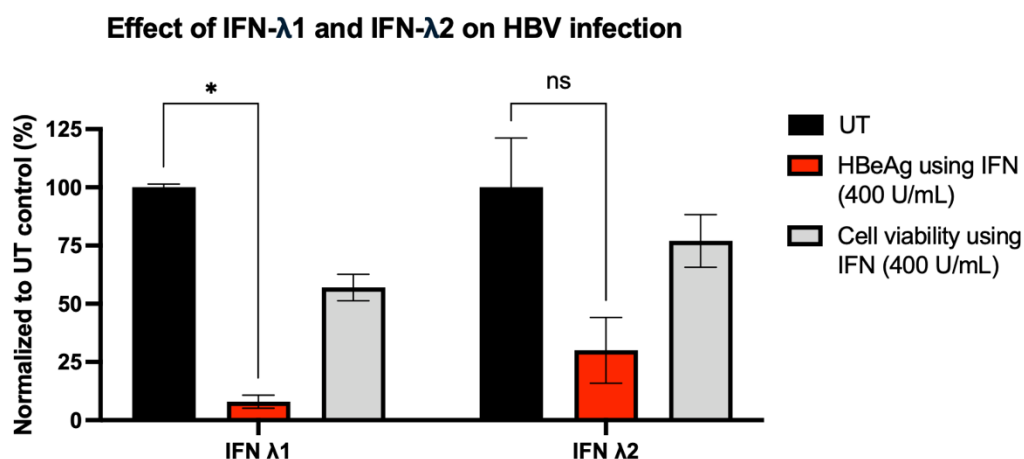


Figure 34: Type III IFNs versus HBV using 500 mge for the infection. HepG2-NTCP B11 were infected with HBV and 16 hours later treated with IFN (400U/mL). IFN treatment was refreshed every 3 days, and the results were assessed by HBeAg ELISA 12 days post-infection. Data are presented as mean $\pm$ SD. The Tukey's multiple comparisons test was used for statistical analysis (ns: not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

After these preliminary results, we considered the limitations of relying on a monoclonal cell line. Although the HepG2-NTCP-B11 cell clone was selected based on its high susceptibility to HBV infection, it may also display distinct, clone-specific features that do not fully reflect physiological cellular responses, especially under interferon treatment. In this context, the strong selection for viral entry and replication could come at the expense of interferon responsiveness, potentially impairing the ability of this model to faithfully recapitulate antiviral IFN activity. Consequently, we expanded our investigation by including a polyclonal cell line to guarantee a more comprehensive evaluation of IFN activity. In particular, we used the parental cell line of the clone B11, known as HepG2-NTCP Pool. This cell population provides a more representative model for assessing antiviral responses because it maintains cellular heterogeneity.

In *Figure 35* is shown the evaluation of the antiviral efficacy of all IFNs previously tested in the HepG2-NTCP Pool cell line. The treatment included two type I interferons (IFN- α 2a and IFN- α 14) and two type III interferons (IFN- λ 1 and IFN- λ 2).

The antiviral effect was measured by evaluating the reduction in HBeAg levels relative to the untreated condition. Notably, IFN- α 14 induced the most substantial reduction in HBeAg levels, indicating a greater efficacy in suppressing HBV replication compared to the other IFNs tested. Indeed, treatment with IFN- α 2a, IFN- λ 1, and IFN- λ 2 resulted in minimal or no detectable decrease in viral markers. These findings further support IFN- α 14 as the most effective interferon for *in vitro* HBV inhibition. Importantly, cell viability was not affected under any of the treatment conditions, confirming that the observed antiviral effects were not attributable to cytotoxicity.

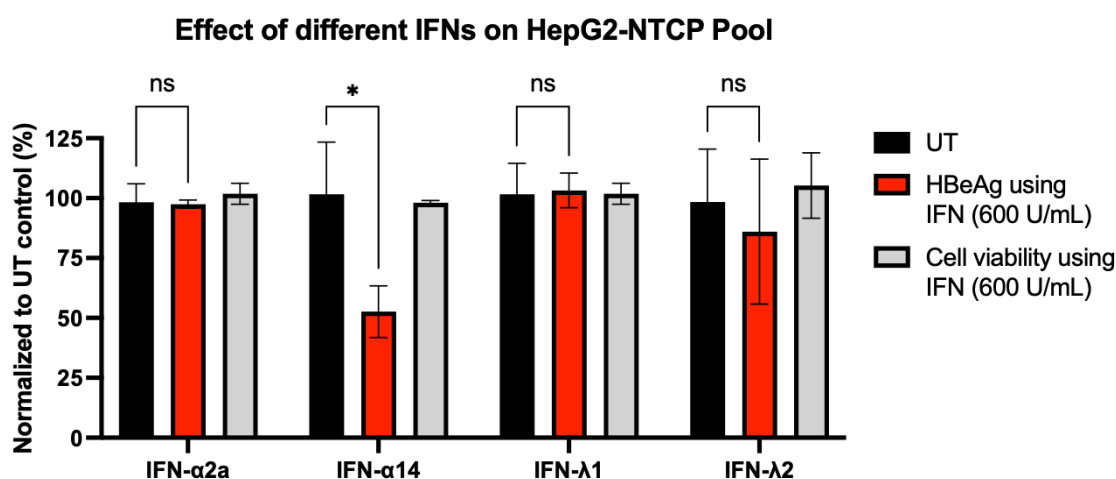


Figure 35: Evaluation of different IFNs on HepG2-NTCP Pool. The treatment consisted of two administrations in a single dose for each IFN of 600U/mL: one following infection and a second refresh dose three days after the initial treatment. Results were assessed by HBeAg ELISA 6 days post-infection and compared to untreated (UT). Data are presented as mean $\pm$ SD. Tukey's multiple comparisons test was used for statistical analysis (ns: not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Nevertheless, the reduction achieved in the HepG2-NTCP Pool reaches a maximum effect of approximately 50%. Therefore, this cellular model cannot be considered optimal to perform a CRISPR-Cas9-based screening.

Although the use of different approaches led to some improvement in assessing the effects of IFNs *in vitro*, the reduction in HBV replication observed in HepG2-NTCP models remained modest and insufficient to fully recapitulate the clinical setting, where IFNs exert a measurable antiviral effect. Indeed, the antiviral activity detected in this system was

limited. Nevertheless, among all IFNs tested, IFN- α 14 consistently showed the strongest inhibitory effect.

In light of these results obtained from infections in the HepG2-NTCP cell lines, we decided to explore alternative approaches, including the use of different methodologies and additional cell lines. This strategic shift aims to overcome the limitations observed and to further characterize other *in vitro* systems that could be suitable for the CRISPR-Cas9 screening.

4.1.3-Huh 7 NTCP and Huh 7.5 NTCP Cells as Surrogate Models for HBV Infection

Developed in 1982 from a human hepatocellular carcinoma (HCC), the Huh 7 cell line has emerged as a crucial model for studies related to the liver, including HBV (Puttahanumantharayappa et al. 2021). The derivative Huh 7.5 cell line, which incorporates a specific mutation in the RIG-I (DDX58) gene, exhibits enhanced permissiveness to viral infections, making it particularly valuable for viral hepatitis research (Kawamoto et al. 2020). Both cell lines were engineered in-house using lentiviral vectors encoding human NTCP, the receptor required for HBV entry. This resulted in stable integration of NTCP into the genome, enabling the cells to support HBV infection.

Although the addition of NTCP, these cells exhibit low susceptibility to HBV infection, but they are still capable of supporting robust viral replication once entry occurs. In fact, both cell lines can replicate viral genomes efficiently, but their infection rates remain low due to inefficient viral entry.

Knowing this limitation, we explored alternative virus delivery approaches in Huh 7 and Huh 7.5 cells:

1. Transfection of plasmids containing a 1.1× HBV genome, a well-established method in which the plasmid is converted by host machinery into cccDNA within the nucleus.
2. RNA launch, which involves intracellular delivery of *in vitro*-transcribed pregenomic HBV RNA (pgRNA). The pgRNA is encapsidated, reverse-transcribed into rcDNA, and converted into cccDNA, recapitulating the viral life cycle (Yu et al. 2023).

Both methods efficiently generate intracellular cccDNA, which is responsible for sustained viral replication, avoiding the viral entry steps, which are inefficient in Huh 7 and Huh 7.5. This makes them particularly suitable for studying post-entry events, cccDNA formation dynamics, and antiviral interventions.

4.1.3.1-1.1X HBV Transfection as a Replication Model Poorly Responsive to IFNs

The 1.1× HBV construct consists of a 1.1X HBV genome placed under the control of a CMV promoter, with transcription starting at nucleotide (nt) 1807. However, the transcription start site for the PreCore mRNA, which is responsible for HBeAg expression, is located upstream, at nt 1785. As a result, in the 1.1× construct, the PreCore leader sequence is missing and cannot be transcribed directly from the plasmid. This sequence is only restored upon cccDNA formation (Zhou et al. 2006). Therefore, HBeAg production from the 1.1× construct strictly depends on successful cccDNA establishment, making it a reliable and functional surrogate marker for cccDNA generation.

By comparing HBeAg levels between the two cell lines following plasmid transfection, we aim to determine which model is more suitable for downstream studies involving 1.1X HBV transfection. *Figure 36* shows the quantification of HBeAg production in Huh 7 NTCP and Huh 7.5 NTCP, following 1.1X HBV transfection.

These results demonstrate that both cell lines are capable of expressing the target antigen. However, the graph clearly indicates a significantly higher level of HBeAg production in Huh 7 NTCP cells compared to Huh 7.5 NTCP cells.

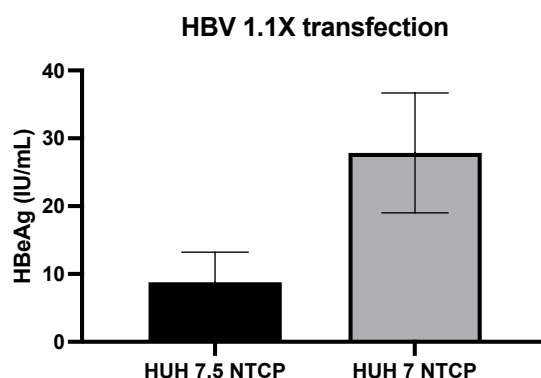


Figure 36: 1.1X HBV transfection in Huh 7 and 7.5. The cell lines were seeded and transfected with 0.5 μ g of 1.1X construct. 6 days post-transfection, results were assessed by HBeAg ELISA. Data are presented as mean $\pm$ SD.

We decided to proceed exclusively with the Huh 7-NTCP cells for further experiments, since they proved to be more responsive to 1.1X transfection. Given that our primary aim was to reproduce the antiviral activity of IFNs *in vitro*, we next designed an experiment to evaluate the effects of four different interferons: IFN- α 2a, IFN- α 14, IFN- λ 1, and IFN- λ 2.

Figure 37 demonstrates that all four interferons tested led to a reduction in HBeAg levels compared to the corresponding untreated controls. Furthermore, cell viability remained consistent across all tested conditions, indicating that the treatment was not cytotoxic. Among the tested IFNs, IFN- α 14 appeared to exert the strongest suppressive effect, showing the lowest HBeAg signal. This result is consistent with previous observations, where IFN- α 14 consistently emerged as the most effective interferon among those tested, even in other experimental contexts.

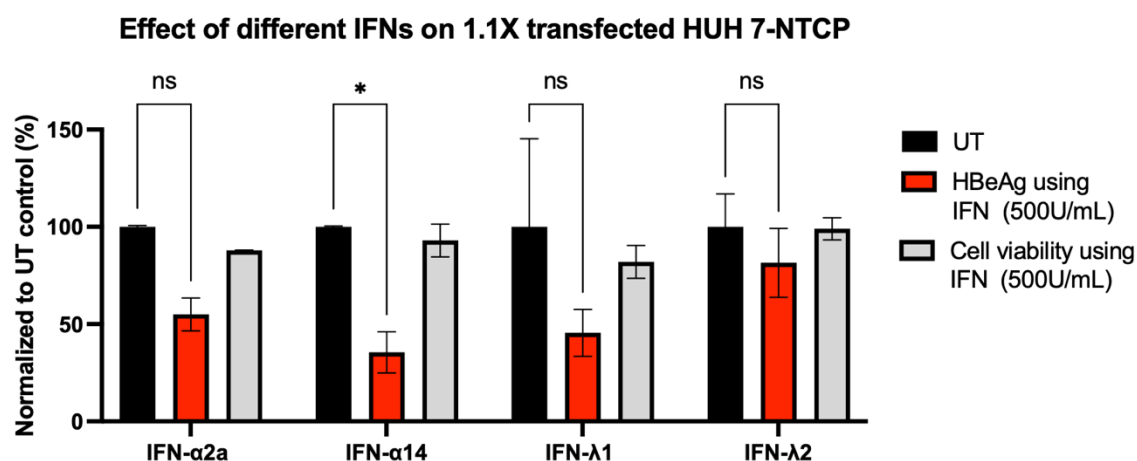


Figure 37: 1.1X HBV Transfection using different IFN treatments. 6 hours after 1.1X transfection, Huh 7-NTCP cells were treated with a single dose of each IFN (500U/mL). 3 days post-transfection, an IFN treatment refresh was performed. Results were assessed 6 days post-transfection through HBeAg ELISA and compared to the untreated condition (UT). Data are presented as mean $\pm$ SD. Sidak's multiple comparisons test was used for statistical analysis (ns: not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

However, despite the observed reduction, the residual signal of viral replication remains relatively high in this setting, with HBeAg levels still around 35%. This is considerably above the desired threshold, as our goal is to achieve near-complete suppression of the signal.

Therefore, we shifted towards alternative experimental approaches in order to achieve a more pronounced inhibition of viral replication.

4.1.3.2-RNA Launch

4.1.3.2.1- HBV Replication from Synthetic RNA through the RNA Launch Strategy

The RNA Launch method is a novel system designed to initiate HBV replication in cell culture using *in vitro*-transcribed pregenomic RNA (pgRNA). Traditional approaches rely on viral infection or plasmid DNA transfection, but both of them introduce background contamination from input viral DNA. The RNA launch is a valid alternative that completely avoids DNA carryover. The viral life cycle is started by transfecting cells with synthetic HBV pgRNA, which is then translated into viral proteins, reverse-transcribed into DNA by the viral polymerase, and finally produces covalently closed circular DNA (cccDNA). This technique enables the study of HBV replication with high signal-to-noise and is particularly useful for evaluating antiviral drugs. Another advantage of the RNA launch approach is the

possibility of utilizing different HBV sequences , such as those from different genotypes, even in the absence of available infectious virus. This flexibility allows for comparative studies on genotype-specific viral behavior and treatment responses. In our experiments, we focused on genotype A (GtA), which is reported to be significantly more sensitive to interferon therapy compared to other HBV genotypes (Yu et al. 2023).

We first optimized the *in vitro* transcription protocol to produce high-quality, capped and polyadenylated HBV pgRNA. After transcription, we determined the optimal pgRNA dose for transfection into Huh 7.5 NTCP cells to ensure a consistent replication signal. Since HBsAg is not directly translated from pgRNA, its detection is considered an indirect marker of successful cccDNA formation. This is because HBsAg expression occurs only if reverse transcription and nuclear import of viral DNA lead to the establishment of cccDNA.

We aimed to determine the optimal amount of HBV RNA required to achieve maximal replication efficiency. As a first step, we assessed the performance of the RNA launch system by transfecting increasing amounts of HBV GtA pgRNA, and quantifying HBsAg production as readout. *Figure 38* shows a dose–response curve generated using HBV GtA pgRNA amounts ranging from 0 to 3.2 μ g. HBsAg levels increased in a dose-dependent manner up to 0.4 μ g of RNA, beyond which no further enhancement in HBsAg production was observed. In fact, higher doses resulted in reduced HBsAg levels, this could be due to saturation of the signal or cytotoxic effects. These results indicate that RNA doses between 0.2 μ g and 0.4 μ g are optimal for achieving robust HBV replication.

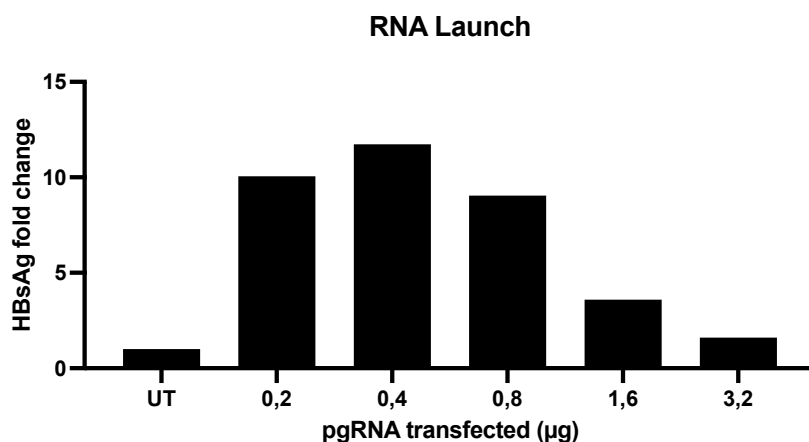


Figure 38: Dose-dependent effect of HBV GtA pgRNA on HBsAg production. Huh 7.5 NTCP cells were transfected with amounts of HBV GtA pgRNA ranging from 0 to 3,2 μ g. 6 days post-transfection, results were assessed through HBsAg ELISA.

4.1.3.2.2- Assessment of IFN Antiviral Efficacy through the RNA Launch Method

Following the identification of the optimal pgRNA dose for the RNA launch, we introduced IFN treatment to assess whether the replication signal could be suppressed. Based on our previous findings, we tested IFN- α 2a, which is clinically approved, as well as IFN- α 14, which had shown the most promising antiviral activity across various *in vitro* models and experimental approaches. A dose-response curve was performed by applying a post-treatment protocol and HBsAg levels were measured to evaluate the impact of interferon treatment on viral replication.

Figure 39 illustrates the RNA launch of HBV GtA pgRNA in Huh 7.5 NTCP cells treated with increasing concentrations of IFN- α 2a. IFN- α 2a treatment did not result in a significant reduction of HBsAg levels at any of the tested concentrations, indicating a limited impact of IFN- α 2a on HBV replication under these experimental conditions. In addition, cell viability remained unaffected across all IFN- α 2a doses, confirming that the treatment was not cytotoxic at the tested concentrations. These results suggest that, despite its clinical use, IFN- α 2a does not provide an antiviral effect in this *in vitro* RNA launch model.

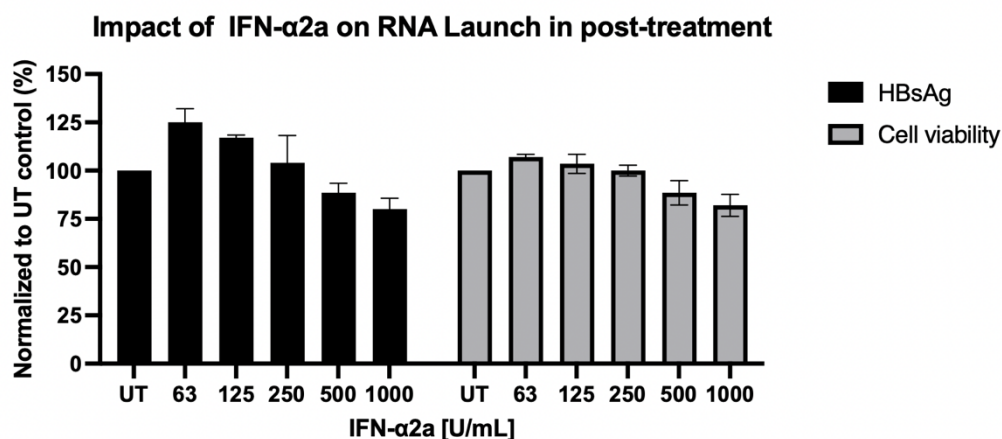


Figure 39: Effect of IFN- α 2a on RNA Launch of HBV GtA. A dose-response curve was established by applying a post-treatment protocol: cells were transfected with pgRNA using HBV GtA and treated twice with IFN, 6 hours post-transfection and 3 days post-transfection. HBsAg levels were measured 6 days after transfection to evaluate the impact of interferon treatment on viral replication. Data are presented as mean $\pm$ SD. Differences were not statistically significant.

Figure 40 shows the RNA launch of HBV GtA pgRNA in Huh 7.5 NTCP cells following treatment with increasing concentrations of IFN- α 14. In contrast to IFN- α 2a, IFN- α 14 treatment led to a modest but consistent reduction in HBsAg levels, suggesting an improved antiviral effect. Cell viability remained relatively stable across all tested conditions. IFN- α 14 exhibited a decrease in HBV replication that was detectable at lower doses compared to IFN- α 2a, where no reduction in HBsAg was observed. After the consistent results obtained in various experimental models, we could confirm the potent antiviral activity of IFN- α 14.

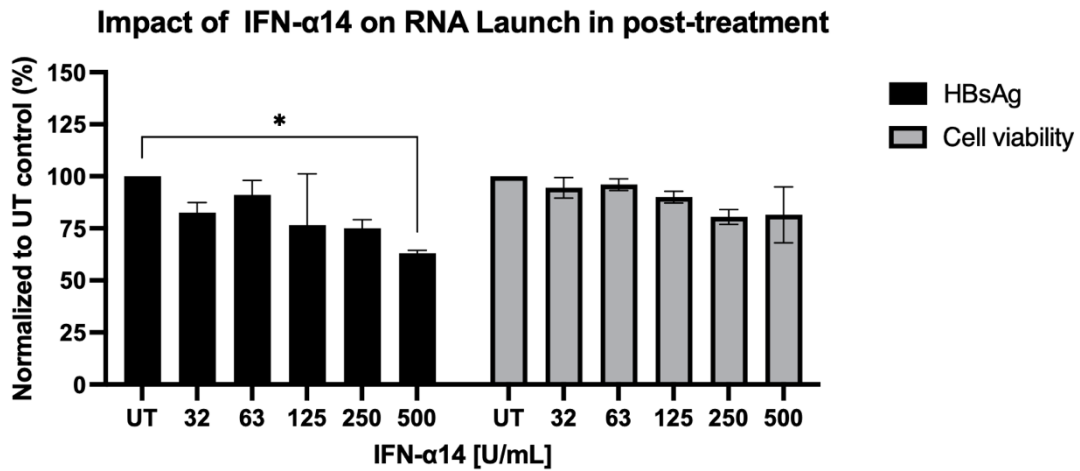


Figure 40: Effect of IFN- α 2a on RNA Launch of HBV GtA. A dose-response curve was established by applying a post-treatment protocol: cells were transfected with pgRNA using HBV GtA and treated twice with IFN 6 hours post-transfection and 3 days post-transfection. HBsAg levels were measured 6 days after transfection to evaluate the impact of interferon treatment on viral replication. Data are presented as mean $\pm$ SD. Tukey's multiple comparisons test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

However, despite the observed reduction in replication signal, the residual HBsAg signal was too high (approximately 60%) to perform a CRISPR-Cas9 screening under these conditions.

To overcome the limits of our *in vitro* model, we decided to evaluate whether pre-treatment with interferon could exert a stronger impact on HBV replication. However, this setup does not fully reflect a physiologically relevant infection model, as cells are treated prior to viral introduction. As shown in Figure 41, the pre-treatment using IFN- α 2a resulted in a reduction of HBsAg levels. Notably, at the highest concentration tested, HBsAg levels dropped to approximately 17%. Importantly, cell viability remained unaffected across all tested doses, indicating that the treatment does not exert significant cytotoxic effects within this concentration range.

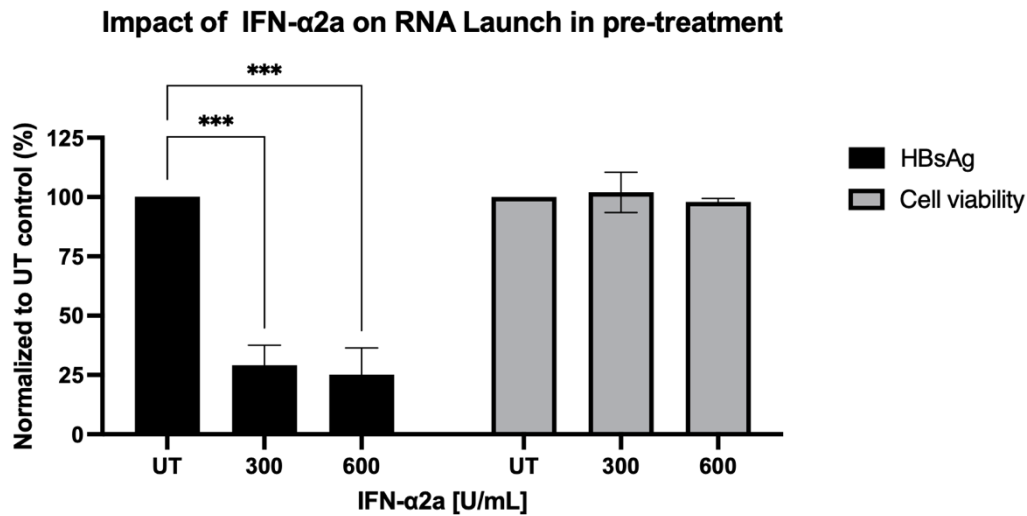


Figure 41: Pre-treatment of IFN- α 2a on RNA Launch of HBV GtA. A dose-response curve was established by treating cells 24 hours before HBV GtA pgRNA transfection. HBsAg levels were measured 6 days after transfection to evaluate the impact of interferon treatment on viral replication. Data are presented as mean $\pm$ SD. Sidak's multiple comparisons test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

We also tested IFN- α 14 in the same conditions, and *Figure 42* demonstrates that this IFN induced a more pronounced suppression of HBsAg levels, indicative of a stronger inhibitory effect on HBV replication. In fact, at the highest concentration tested, HBsAg levels dropped to approximately zero.

The superior antiviral effect of IFN- α 14 was already evident at lower concentrations because even at a concentration of 200 U/mL, HBsAg levels was reduced to 3%. This demonstrates that IFN- α 14 has a more potent antiviral response even at lower doses compared to IFN- α 2a. Importantly, cell viability remained unaffected across all treatment conditions, similarly to what was observed with IFN- α 2a. This confirms that IFN- α 14's enhanced antiviral activity is not due to cytotoxicity.

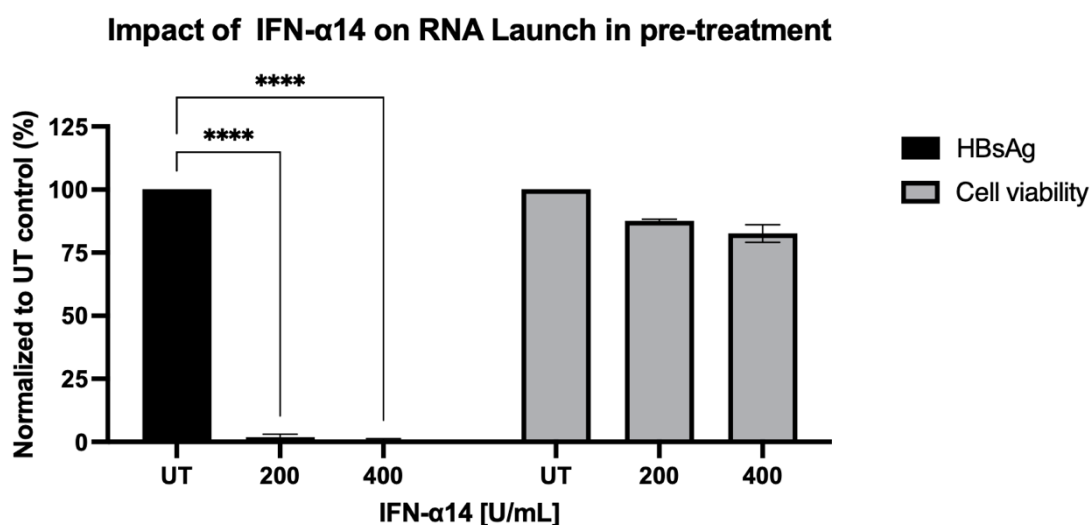


Figure 42: Pre-treatment of IFN- α 14 on RNA Launch of HBV GtA. A dose-response curve was established by treating cells 24 hours before HBV GtA pgRNA transfection. HBsAg levels were measured 6 days after transfection to evaluate the impact of interferon treatment on viral replication. Data are presented as mean $\pm$ SD. Tukey's multiple comparisons test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.1.3.2.3- RNA Launch Limitations

However, we had to consider that IFN might affect and reduce the expression of any foreign RNA, regardless of its origin. Indeed, the fact that the IFN treatment was administered before transfection increases the possibility that the decrease assessed may not be specific to HBV. Indeed, ISGs can broadly inhibit translation, degrade foreign RNA, or activate innate immune pathways that target a wide range of RNAs. We conducted a control experiment in which cells were pre-treated with IFN and then transfected with a GFP-encoding mRNA to see whether the observed decrease in expression was specific to our RNA of interest or rather a general effect of IFN activation. We chose to use GFP as its mRNA is non-viral and not targeted by particular antiviral mechanisms.

Figure 43 shows the percentage of GFP-positive cells under three experimental conditions: no IFN treatment, IFN treatment post-transfection, and IFN pre-treatment before the transfection. While IFN therapy after transfection has a negligible impact on GFP expression, IFN pre-treatment dramatically reduces the number of GFP-positive cells. The reduction observed following IFN pre-treatment suggests that the effect observed with HBV is non-specific and can be related to all RNA transfections. Therefore, the reduced signal we

observed with HBV RNA launch after IFN pre-treatment is more likely correlated to an IFN-induced suppression of transfected RNA rather than a virus-specific response.

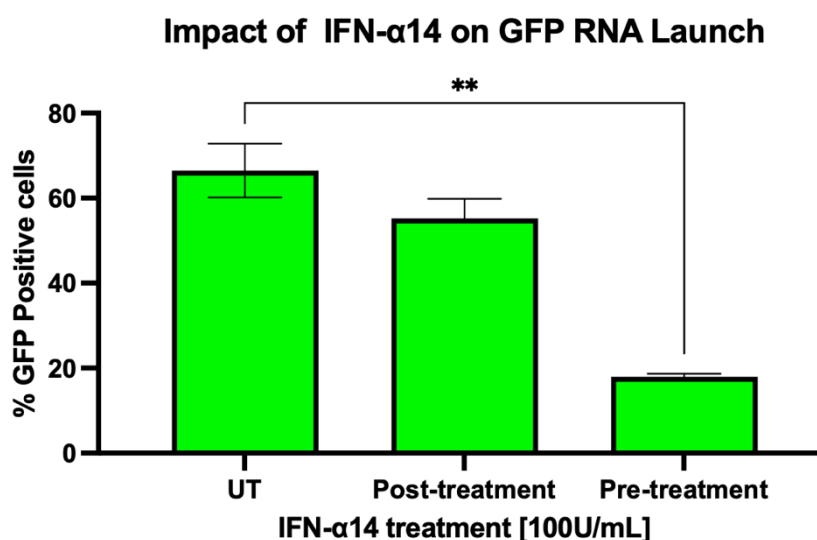


Figure 43: mRNA GFP transfection using IFN treatment. Huh 7.5 NTCP cells were treated both in pre-treatment (24 hours before transfection) and in post-treatment (6 hours after transfection). Results were assessed 3 days post-transfection through flow cytometry, and both the IFN conditions were compared to the untreated (UT). Data are presented as mean $\pm$ SD. Tukey's multiple comparisons test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

In light of these findings, we decided to discontinue the RNA launch approach. This conclusion is further supported by the absence of a comparable inhibitory effect when IFN is administered after transfection, highlighting the critical impact of timing on IFN activity. Together, these observations indicate that the RNA launch model is not suitable for our purpose.

4.1.4- DMSO Influence on HBV Infection and IFN Response

PHHs represent the most physiologically relevant *in vitro* model of liver function and metabolism, as they closely reproduce the characteristics of the human liver. Despite their status as the gold standard, their use is hindered by limited availability, donor-dependent variability, lack of proliferative capacity, and short life. In contrast, the hepatoma-derived HepG2 cell line is widely employed in metabolic studies but exhibits altered metabolic features due to its malignant origin, including elevated de novo lipogenesis, low triglyceride secretion, and elevated glycolysis (Daniëls et al. 2014). Dimethyl sulfoxide (DMSO), is a molecule known to influence diverse cellular processes and has been reported to promote differentiation and drug-metabolizing activity in hepatoma cell lines (Choi et al. 2009).

Supplementation of HepG2 cultures with 1% DMSO induces a shift toward a metabolic phenotype that more closely resembles PHH, characterized by enhanced fatty acid oxidation, triglyceride secretion, and increased gluconeogenesis. This approach therefore provides an improved *in vitro* model for studying hepatocyte biology, liver pathophysiology, and therapeutic responses (Nikolaou et al. 2016).

Given this evidence, HepG2-NTCP infection is usually carried out in the presence of 2.5% DMSO. This concentration is then maintained throughout the entire experimental period. For this reason, we decided to seed our HepG2-NTCP B11 cells directly in culture medium containing 2.5% DMSO. The rationale was to assess whether such conditions could induce cellular differentiation, thereby enhancing their responsiveness to IFN and promoting a phenotype closer to PHH.

Dose-response analysis of IFN- α 14 revealed a striking difference in antiviral efficacy between cells cultured with or without DMSO (*Figure 44*). In the absence of DMSO, IFN- α 14 treatment resulted only in a partial reduction of HBV antigen levels, indicating limited antiviral activity. By contrast, when cells were plated in medium complemented with DMSO, IFN- α 14 elicited a markedly enhanced response, with antigen levels reduced to approximately 15% of the untreated control. This represents a substantial improvement over previous conditions and underscores the capacity of DMSO to potentiate the antiviral effect of IFN- α 14. Importantly, parallel assessment of cell viability using the CTB assay demonstrated no adverse effects associated with DMSO treatment or IFN exposure, confirming that the observed reduction in HBV antigen reflects a specific antiviral response rather than cytotoxicity.

Effect of DMSO on IFN-Mediated Antiviral Activity

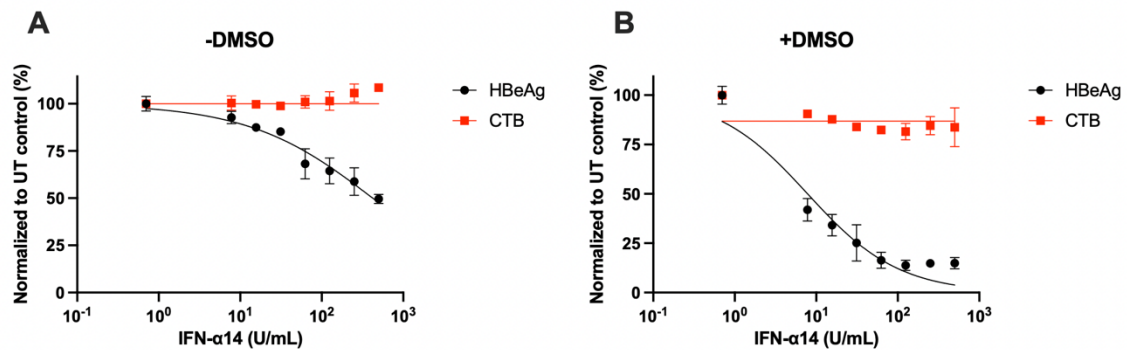


Figure 44: Comparison between HepG2-NTCP B11 seeded without (A) and with (B) 2,5% DMSO and treated with IFN- α 14. Cells were plated with or without DMSO and 24 hours later were infected with 500 mge of HBV. 16 hours post-infection, both conditions were treated with IFN- α 14. IFN treatment was refreshed following 3 days, and the results were assessed by HBeAg ELISA 7 days post-infection. Data are presented as mean $\pm$ SD.

While DMSO appears to act as the critical determinant of the markedly enhanced antiviral activity of IFN- α 14, these findings require further validation through a direct comparison of infection levels between the different culture conditions. It remains possible that the observed outcome does not exclusively reflect a stronger antiviral effect, but rather a reduced susceptibility of the cells to HBV infection under DMSO treatment.

To assess whether DMSO treatment itself could influence the susceptibility of cells to HBV infection, we compared infection rates following a 24-hour exposure to DMSO. Infection was evaluated by quantifying HBeAg levels. As shown in *Figure 45*, both conditions exhibited similarly high levels of infection. A modest reduction in signal was observed in cells cultured with DMSO; however, this difference was minimal and is more likely attributable to a reduced number of target cells rather than to a true impairment of viral infection.

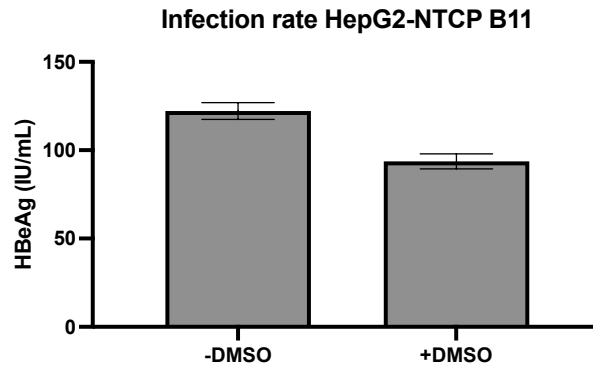


Figure 45: Infection rate of HepG2-NTCP B11 plated without and with 2,5% DMSO. Cells were infected 24 post-seeding with 500 mge of HBV. Results were assessed by ELISA HBeAg 7 days post-infection and compared using the amount of international units (IU) generated. Data are presented as mean $\pm$ SD.

These results indicate that the enhanced antiviral effect of IFN- α 14 under DMSO treatment is not due to differences in baseline infection. The HBV inhibition observed is instead attributable to the 24-hour DMSO exposure before infection. We therefore decided to use this condition to test additional infection conditions by increasing the viral inoculum and assessing whether the effect of IFN- α 14 would be maintained or diminished.

In order to evaluate whether the antiviral activity of IFN- α 14 was maintained under conditions of high viral input, we performed infection experiments using elevated HBV inoculum of 1000 and 5000 mge. HBV replication was assessed by quantifying viral antigen levels, while cell viability was simultaneously monitored by the CTB assay. As shown in *Figure 46*, treatment with IFN- α 14 resulted in a pronounced reduction of HBV antigen signal in both infection settings, indicating that its antiviral efficacy is preserved even at high viral loads. Importantly, CTB measurements remained comparable across conditions, confirming that IFN- α 14 treatment did not compromise cell viability.

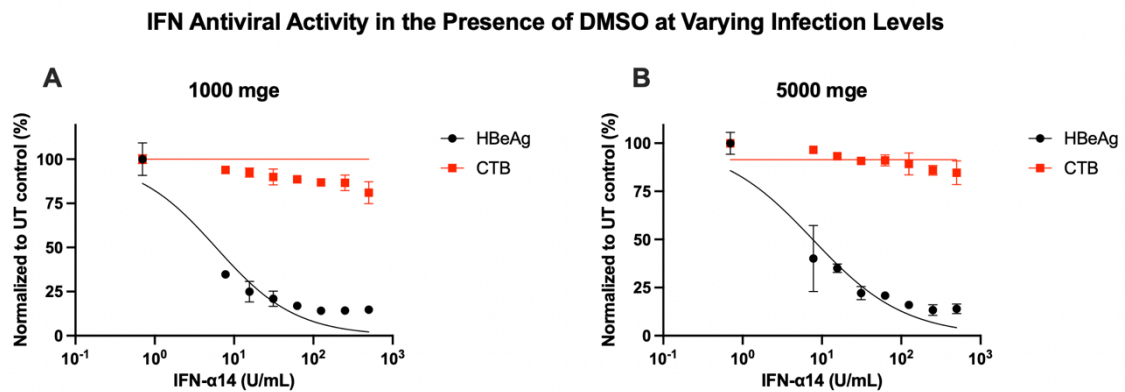


Figure 46: IFN- α 14 effect using 1000 mge (A) and 5000 mge (B) of HBV. HepG2-NTCP B11 were plated with 2,5% of DMSO and 24 hours later were infected with HBV. 16 hours post-infection, both conditions were treated with IFN- α 14. IFN

treatment was refreshed following 3 days, and the results were assessed by HBeAg ELISA 7 days post-infection. Data are presented as mean $\pm$ SD.

Having established that IFN- α 14 preserved its antiviral activity even under conditions of elevated HBV inoculum, we next aimed to investigate whether this effect was unique to this particular subtype or could be generalized to other IFN- α . To address this, we tested the efficacy of IFN- α 2a, which has been consistently used as a reference standard in our experiments. Therefore, we evaluated the antiviral activity of IFN- α 2a under the same inoculum infection conditions (500 mge), monitoring both HBeAg and cell viability. As shown in *Figure 47*, treatment with IFN- α 2a resulted in a reduction of viral antigen signal, confirming the antiviral effect. However, the potency of this effect was clearly lower compared to that observed with IFN- α 14 under identical conditions. In particular, while IFN- α 14 induced a pronounced decrease in HBeAg levels even at high viral inputs, IFN- α 2a achieved only a limited suppression, suggesting a reduced antiviral effect in this context.

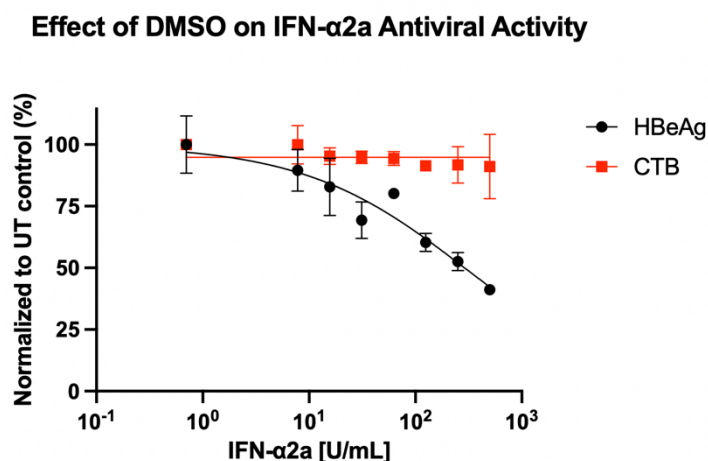


Figure 47: IFN- α 2a effect using 500 mge of HBV. HepG2-NTCP B11 were plated with 2,5% of DMSO and 24 hours later were infected with HBV. 16 hours post-infection, both conditions were treated with IFN- α 2a. IFN treatment was refreshed following 3 days, and the results were assessed by HBeAg ELISA 7 days post-infection. Data are presented as mean $\pm$ SD.

In conclusion, seeding HepG2-NTCP B11 in the presence of 2,5% DMSO was found to influence the antiviral activity of IFN against HBV only under specific conditions. Notably, this effect appeared to be restricted to IFN- α 14, suggesting that DMSO treatment may drive cellular differentiation towards a phenotype suitable for the IFN- α 14 signaling pathway but not for IFN- α 2a. These results suggest that DMSO pre-treatment confers an antiviral effect

specific to IFN- α 14, while cells remain susceptible to infection. This highlights the complex interplay between cellular differentiation status, IFN subtype specificity, and HBV permissiveness.

4.2- Development of a Method to Select HBV-Positive Cells

Another important goal of the present study was to identify an optimal experimental strategy to select the positive cells after the CRISPR-Cas9 screening. There are several methods to identify HBV-positive cells selectively. We focused on two main strategies: developing a reporter virus and performing a staining using HBcAg.

In order to directly select infected cells based on fluorescence, the initial strategy involved creating a recombinant hepatitis B virus that expresses a fluorescent reporter protein. However, designing an HBV reporter virus is technically very challenging due to the compact organization of the viral genome, which is composed of multiple overlapping open reading frames (ORFs). Due to this genomic arrangement, large exogenous sequences, including full-length fluorescent proteins, cannot be introduced without impairing viral capabilities. Infectivity or replication may be compromised even by small insertions that disrupt the genome stability.

Several reporter HBV systems have been developed, mostly relying on luciferase enzymes, such as the HiBiT system, a peptide that, when complemented by a partner subunit, produces luminescence (Sumiyadorj et al. 2022). Despite the signal of these kinds of reporters being reliable with HBV life cycle, luciferase-based reporters are not suitable for CRISPR-Cas9 pooled screens, as they do not enable direct selection of individual infected cells. On the other hand, a fluorescent marker or a selectable gene enables the enrichment of particular cell populations, usually needed for this type of screening.

An alternative strategy we considered was the intracellular staining of a viral antigen expressed within the infected cells, such as HBcAg. This method allows the detection and quantification of HBV-positive cells by flow cytometry. However, this method is more laborious and requires an antibody optimised for intracellular set-ups, which are poorly described in the literature.

4.2.1-GFP-based reporter system

4.2.1.1-Development of a GFP-based reporter system for HBV

To develop our reporter system for HBV, we employed the self-complementing split-GFP technology. This approach takes advantage of two engineered fragments of GFP that spontaneously assemble: a tiny peptide tag called GFP11, which represents the 11th beta strand and a large, non-fluorescent detector fragment GFP1–10, which consists of beta strands 1–10. Sensitive detection of GFP11-tagged proteins is achieved through the natural reassembly of both pieces into a functional fluorescent protein when they are available in the same cellular compartment. This method is especially well-suited when a full-length fluorescent protein is undesirable or technically impractical, such as in the context of small viral genomes or when minimal tag size is crucial, such as in the context of HBV (Avilov and Aleksandrova 2019).

After the evaluation of the best reporter system to use, we had to identify the optimal sequence location to insert our system. We aimed to find a viral protein that is suitable for small tag insertion and that is not necessary for viral replication, to minimise any impairment of viral fitness.

Furthermore, Important cis-acting components in the HBV genome, such as the epsilon (ϵ) sequence, must also be preserved. In fact, the 5' region of the pgRNA contains a conserved RNA secondary structure called the ϵ element, which acts as both the starting point for reverse transcription by the viral polymerase and the packaging signal for encapsidation into nucleocapsids. If this component is disrupted, the viral replication is compromised. Overall, it was important for our study to extensively investigate the most strategic location to insert this protein without impairing any fundamental component of the HBV genome.

Given all these informations we chose to engineer HBeAg. *Figure 48* illustrates the strategy used to engineer the HBeAg sequence. We inserted our sequence of interest, containing GFP11, immediately upstream of the core protein's start codon (ATG), in order to avoid disrupting the core protein, which is essential for HBV replication. Importantly, this insertion was designed to preserve the integrity of the epsilon (ϵ) structure and the Kozak sequence right before the HBcAg start codon. To achieve this, we included an additional sequence upstream of the GFP11 tag to reconstruct and maintain the correct structure of the ϵ element.

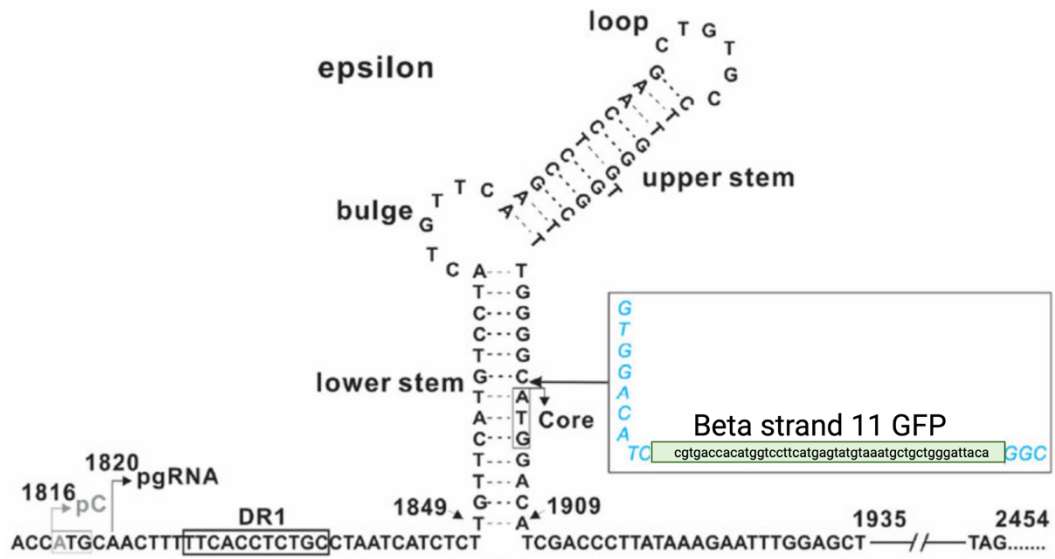


Figure 48: HBeAg engineering. We inserted the peptide tag called GFP11 right before the ATG of HBeAg. This peptide is flanked by elements that reconstitute the epsilon and preserve the architecture of the HBV genome.

However, since HBeAg is mostly secreted outside the cell, this could limit the efficiency of GFP folding, as GFP1-10 is retained within the intracellular compartment. Anyway, the synthesis of HBeAg requires several intracellular precore-derived intermediates that temporarily accumulate within the cell, even though the protein is ultimately released from infected cells. An N-terminal signal peptide directs the cytoplasmic synthesis of the full-length precore precursor (p25) into the endoplasmic reticulum (ER) (Garcia et al. 1988). P25 is then converted into p22, a crucial intermediate that remains temporarily in the ER. Further processing in the trans-Golgi network generates the mature, secreted HBeAg (p17). However, not all the intermediates successfully follow this pathway. Phosphorylated cytosolic forms (P-p25 and P-p22) are produced when a component of p25 and p22 undergoes abortive translocation or retrotranslocation into the cytoplasm. It has been demonstrated that these cytosolic species can form chimeric capsids with HBV core protein (HBc) and can remain within hepatocytes, indicating that they are accessible and structurally functional in the intracellular environment (Hong et al. 2023). Even though HBeAg is secreted extracellularly, the presence of these intracellular intermediates suggested that GFP11 fused to the precore protein could supplement the cytosol-retained GFP1-10, allowing fluorescence signal recovery.

We therefore developed six constructs based on the 1.1X HBV plasmid. As previously described, in this plasmid, the formation of cccDNA is a necessary condition for HBeAg production. Consequently, by using this system, we can ensure that the complementation signal leading to fluorescent GFP formation is directly dependent on cccDNA.

In *Figure 49*, the six versions of the HBV 1.1X construct that we developed are shown, each modified to include the β -strand 11 of GFP. These versions differ from one another in the linker sequences surrounding GFP11 or in the presence and position of stop codons downstream of GFP11.

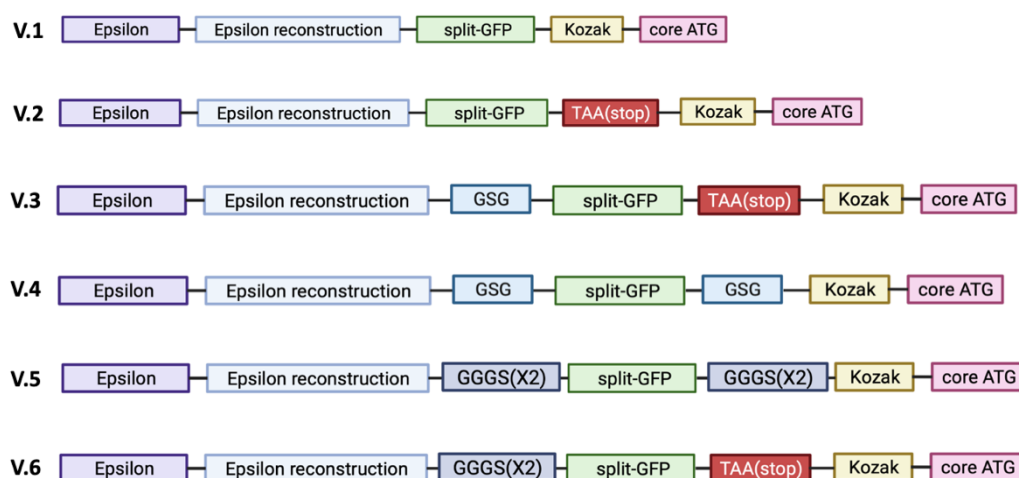


Figure 49: 1.1X HBV reporter versions. V1-V6 are represented only in the part of the genome that is engineered. V2, V3, and V4 have a stop codon right after the GFP11 peptide; therefore, they produced a truncated form of HBeAg. V1, V4, and V5 result in the production of the full HBeAg.

4.2.1.2-Assessment of HBV Reporter Replication Efficiency

Figure 50 illustrates the assessment of the replication efficiency of the six different HBV 1.1X plasmid variants. HepG2 NTCP B11 were transfected with each 1.1X version, and HBV genomic material in the supernatant was quantified using real-time PCR. As shown in the graph, all plasmid variants resulted in a detectable HBV DNA signal, indicating successful initiation of replication. To ensure that the detected HBV signal originated from the actual replication process and not from residual input plasmid DNA, we included a real-time PCR amplification of the ampicillin resistance gene (Amp) present on the plasmid backbone. For all variants, the Amp signal was significantly weaker compared to the HBV signal. The difference clearly indicates that the HBV DNA detected is the result of active HBV replication initiated after transfection, rather than plasmid contamination.

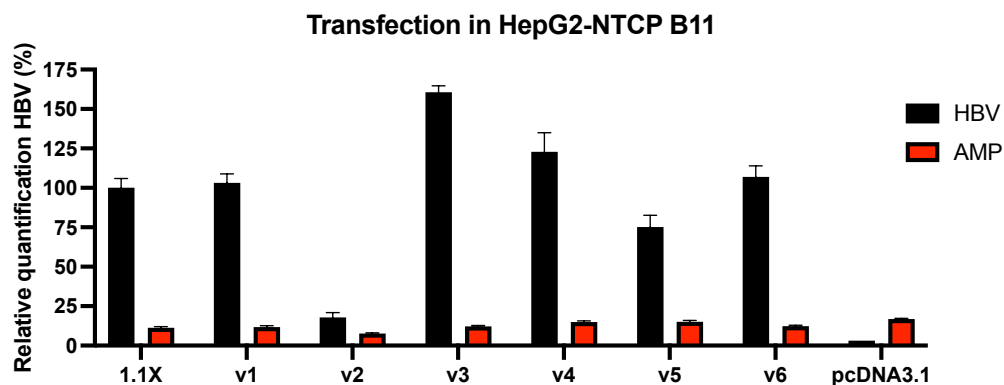


Figure 50: Replication signal of HBV reporter viruses. Each 1.1X variant was transfected into HepG2-NTCP B11, and the results were quantified through real-time PCR 6 days after transfection. As controls, we inserted the ampicillin resistance gene and an empty plasmid with the same backbone as the 1.1X plasmid called pcDNA 3.1, which should result in no detectable signal for HBV. Data are presented as mean $\pm$ SD.

To further confirm this active viral replication signal in *Figure 51*, we performed the same experiment inserting the tenofovir as a control. Tenofovir is a nucleotide analog reverse transcriptase inhibitor (NtRTI) commonly used in the treatment of chronic HBV infection. By functioning as a chain terminator, it inhibits the reverse transcription step necessary for HBV replication, blocking the synthesis of new HBV DNA (Jenh et al. 2009).

As the graph illustrates, the Ampicillin signal stays constant and is insignificant compared to the HBV signal. All six HBV variants exhibit a marked reduction in viral DNA levels after being treated with tenofovir, confirming that they can all start viral replication and that the signal seen in untreated samples is specific to HBV replication and not caused by the background noise of plasmid DNA. These findings show that all six HBV 1.1x reporter constructs are replication-competent in this context and validate the replication signal's specificity.

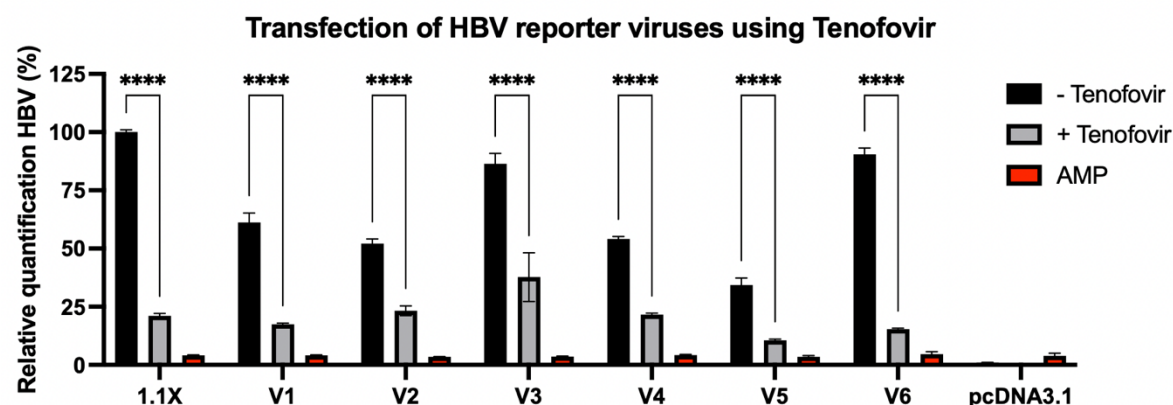


Figure 51: Replication signal of HBV reporter viruses using Tenofovir. Each 1.1X variant was transfected into HepG2-NTCP B11 and treated with Tenofovir [0,1 μ M] 6 hours post-transfection. Results were quantified through real-time PCR 6 days after transfection and compared to the relative AMP and Tenofovir signal. Data are presented as mean $\pm$ SD. Tukey's multiple comparisons test was used for statistical analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.2.1.3-Complementation and Functional Assessment for GFP Reconstitution

After confirming the proper replication capacity of the HBV 1.1x reporter constructs, we aimed to assess the ability of the GFP11 tag inside these constructs to complement with the remaining GFP1–10 fragment and reconstitute fluorescence. To maximize this detection, we first generated plasmids designed exclusively to express the HBeAg, without the whole replication genome. This allowed us to evaluate GFP complementation in isolation, before reintroducing the full 1.1x constructs to produce replication-competent viruses.

To this end, we cloned the precore mRNA sequence, which encodes the HBeAg, into the pcDNA3.1 plasmid backbone for each of the six reporter variants.

In Figure 52 is shown the co-expression of GFP1-10 with the six constructs expressing the HBeAg fused to GFP11, enabling us to test fluorescence reconstitution in a replication-independent system, such as HEK293TN. As a control for complementation efficiency, we included a construct expressing a fully folded GFP, as well as a standard split-GFP system where GFP11 is fused to a clathrin light chain (CLC), a model substrate for this assay.

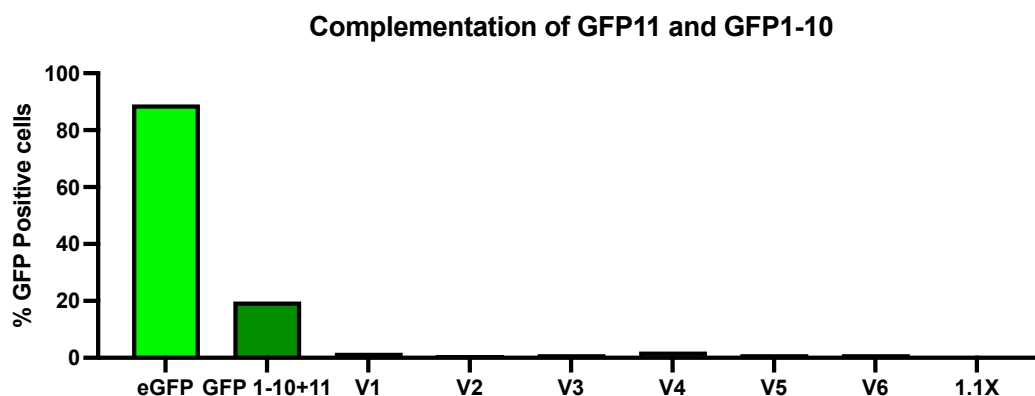


Figure 52: Assessment of GFP complementation in cells transfected with HBeAg-GFP11 fusion constructs. Each construct was co-transfected with GFP1-10 inside HEK293TN cells. Results were evaluated through flow cytometry 4 days post-transfection.

As shown in the graph, all six HBeAg-GFP11 reporter constructs displayed minimal or no detectable GFP complementation when co-expressed with GFP1–10 in HEK293TN cells. Only constructs v1 and v4 exhibited a very faint signal, with GFP-positive cells remaining at extremely low percentages. These results indicate that, under the tested conditions, the split-GFP system is largely inefficient when GFP11 is fused to the HBeAg.

Importantly, even the standard complementation GFP11-CLC control showed modest efficiency, with a maximum of only 20% GFP-positive cells. The results of this control further highlight the weaknesses of the system, especially if we plan to apply it to an HBV infection system for our screening. The fully folded GFP control, on the other hand, demonstrated strong GFP fluorescence in about 90% of transfected cells, as predicted, allowing us to exclude any technical error. These findings imply that the HBeAg-GFP11 fusion protein is not ideal for strong GFP complementation, and system modifications are probably necessary before using the system in a physiologically relevant context.

To further investigate the results of the complementation assay, we focused on confirming that the intracellular portion of HBeAg was both detectable and quantifiable by Western blot analysis. This step was essential to ensure that complementation could occur and that the intracellular forms of the antigen were indeed expressed and present. To evaluate the intracellular forms of HBeAg produced by our constructs, we performed a Western blot using a polyclonal anti-core antibody. Given that the HBcAg and HBeAg share the majority of their amino acid sequence, a polyclonal anti-core antibody is suitable for detecting HBeAg and its intracellular precursors. HEK293TN cells were transfected with various constructs,

including our experimental reporters encoding only for the mRNA of HBeAg (V1, V4, V5, and V6) and HBeAg and its intermediates (p17, p22, and p25) as controls. Among the reporter constructs, V2, V3, and V6 contain premature stop codons, resulting in truncated mRNA and thus no detectable protein; only V6 was included here as a negative control. Constructs p22 and p25 encode intracellular precursors of HBeAg, which are expected to be detectable in cell lysates as they are intracellular intermediates, while p17 represents the mature secreted form of HBeAg, which is not expected to be visible by Western blot. As an additional control, we included p21, expressing the HBV core protein, which the antibody specifically recognizes and should thus produce a strong signal. Actin (ACT) was used as a loading control.

In *Figure 53* are shown the results, and as expected, V6 shows no signal, confirming the absence of protein expression. Reporter constructs V1, V4, and V5 exhibit weak but specific bands, consistent with the presence of intracellular intermediates only. The strong signals observed for p22 and p25 confirm proper detection of the respective intracellular proteins, while the absence of signal for p17 supports the notion that mature HBeAg is secreted and not retained within the cell.

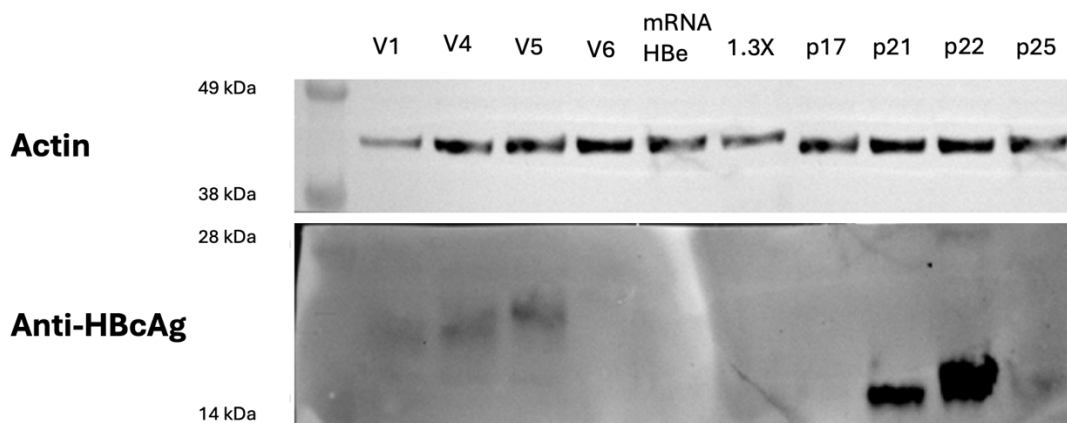


Figure 53: Western blot of reporter constructs and controls. HEK293TN cells were transfected with reporter constructs and intermediates of HBeAg. The Western blot was performed 3 days after transfection using a polyclonal antibody anti-HBcAg.

These results validate the design and function of our constructs and confirm that an intracellular complementation could occur with GFP1-10.

To further assess the expression of the GFP11 β -strand in our constructs, we performed a Western blot using an anti-GFP11 antibody. This experiment aimed to confirm that the GFP11 tag is indeed expressed alongside HBeAg in constructs without premature stop codons, thereby validating the potential for intracellular complementation with GFP1-10. As in the previous blot, HEK293TN cells were transfected with the six constructs (V1 to V6), and actin was used as a loading control.

The results shown in *Figure 54* demonstrate that, although the signal strength is weak, V1, V4, and V5 produce a signal at the expected molecular weight for the GFP11 fused with HBeAg. This weak signal indicates the low number of intracellular HBeAg intermediates and is in line with what was shown with the anti-core antibody. In V2, V3, and V6, which include premature stop codons and should not result in a full-length GFP11-fusion protein, no signal was found. These findings demonstrate that the HBeAg-GFP11 fusion protein can be produced by constructs lacking stop codons (V1, V4, V5), enabling functional complementation with GFP1-10 inside cells.

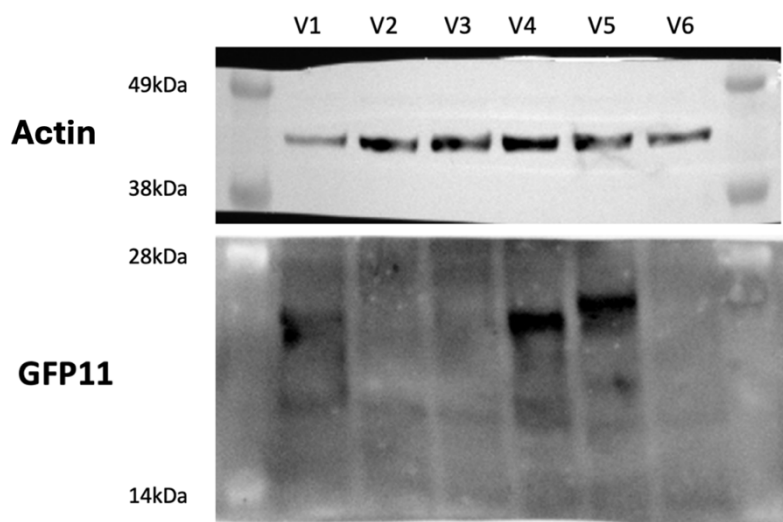


Figure 54: Western blot of reporter constructs. HEK293TN cells were transfected with reporter constructs V1-V6. The Western blot was performed 3 days after transfection using an anti-GFP11 antibody.

Although constructs V2, V3, and V6 do not produce detectable protein by Western blot due to the presence of premature stop codons, they are still valuable for experimental purposes. These constructs are designed to eliminate HBeAg expression while potentially preserving the expression of a truncated form of the protein containing GFP11. This strategy opens the possibility for GFP complementation inside cells, even in the absence of full-length HBeAg,

providing an additional tool for studying intracellular localization and interactions independent of antigen maturation.

To improve the system, we took into consideration that the intracellular portion of HBeAg during maturation is located in the endoplasmic reticulum (ER). We designed the construct with the complementary GFP1-10 fragment, which should interact with the HBeAg-GFP11, to improve its localization to the ER. In particular, we involved two targeting sequences: a C-terminal KDEL motif and an N-terminal signal peptide. The KDEL sequence (Lys-Asp-Glu-Leu) ensures retention of the protein within the ER lumen by preventing its transport to the Golgi apparatus (Chen et al. 2021).

Short, hydrophobic amino acid sequences called signal peptides (SPs) are usually found at the N-terminus of proteins and act to guide them to the ER. A signal peptide works as a trafficking signal when it is attached to a protein, directing it to the ER membrane where it interacts with the signal recognition particle (SRP). Therefore, adding an SP to a protein causes a delocalisation to the ER, inducing the processing of the protein for secretion or membrane insertion (Liaci and Förster 2021).

To test the effect of these sequences, we generated two modified GFP1-10 constructs: one containing only the signal peptide, and another containing both the signal peptide and the KDEL sequence. Therefore, we tested these two new constructs in a complementation assay.

Figure 55 shows HEK293TN cells transiently co-transfected with one of the two GFP1-10 variants and with each of the six HBV reporter constructs. These constructs expressed only HBeAg, fused to the split GFP11 tag. Complementation efficiency was evaluated by quantifying the percentage of GFP-positive cells, indicating successful reconstitution of the fluorescent signal. The results show a clear improvement compared to previous experiments using non-modified GFP1-10. Notably, for all antigen constructs capable of producing a detectable signal, fluorescence was consistently higher in the SP+KDEL condition compared to SP alone.

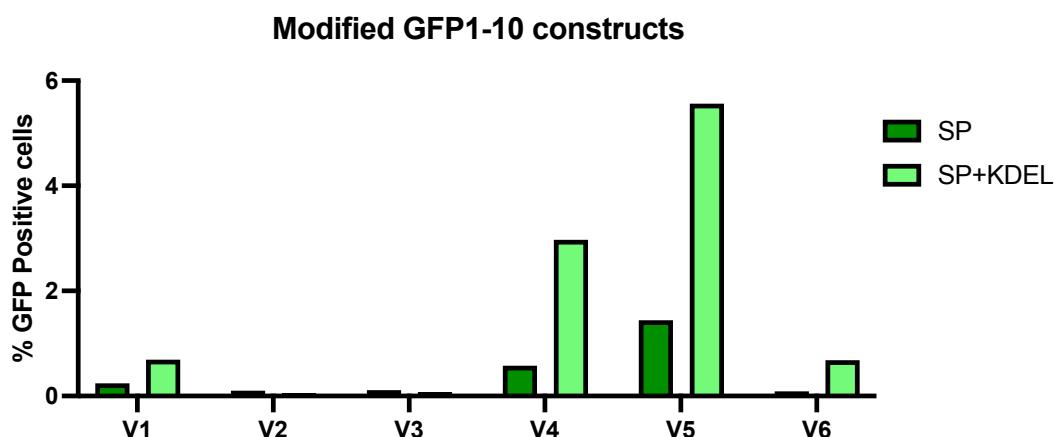


Figure 55: v1-v6 complementation with GFP1-10 new versions. Each construct was co-transfected with each GFP1-10 version inside HEK293TN cells. Results were evaluated through flow cytometry 4 days post-transfection.

This confirms that the strategy to enhance ER targeting and retention was effective and significantly improved the system's performance. The results show that variants V4 and V5 yielded the highest levels of GFP-positive cells, identifying them as the most efficient reporters under these optimized conditions.

Despite the clear improvements achieved with the ER-targeted GFP1-10 constructs, the overall complementation efficiency remains relatively low. In fact, fluorescent signal is detected only in less than 10% of transfected cells, indicating that split GFP reconstitution occurs sporadically.

All the experiments just described were performed in HEK293TN cells; the next step was therefore to test the split GFP system in a cell line more suitable for HBV research. To this end, we aimed to optimize the complementation system using all the information acquired in the previous experiments. To optimize the GFP-based complementation system, we generated stable hepatic cell lines expressing the GFP1-10 fragment modified with both SP and KDEL. The stable expression of GFP1-10 ensures its consistent intracellular presence, thereby maximizing the efficiency of complementation upon transfection with the HBeAg reporter construct. We employed hepatic cell lines for this purpose, which will be the standard model for the infection with the reporter virus. Indeed, we established three stable cell lines via lentiviral transduction: HepG2-NTCP, Huh7-NTCP, and Huh7.5-NTCP, each engineered to express the SP+KDEL-modified GFP1-10.

To first assess their baseline capacity for complementation, we transfected them with plasmids expressing HBeAg fused to GFP11 (constructs V4 and V5). An untreated (UT) control was included to establish background fluorescence levels in the absence of GFP11. The results, as shown in *Figure 56*, were generally disappointing. Only the Huh 7 NTCP line showed a detectable signal above background, with GFP complementation reaching a maximum of less than 2% of positive cells. Both HepG2 NTCP and Huh 7.5 NTCP lines failed to show a significant GFP signal upon transfection, suggesting either poor expression or inefficient complementation in these systems.

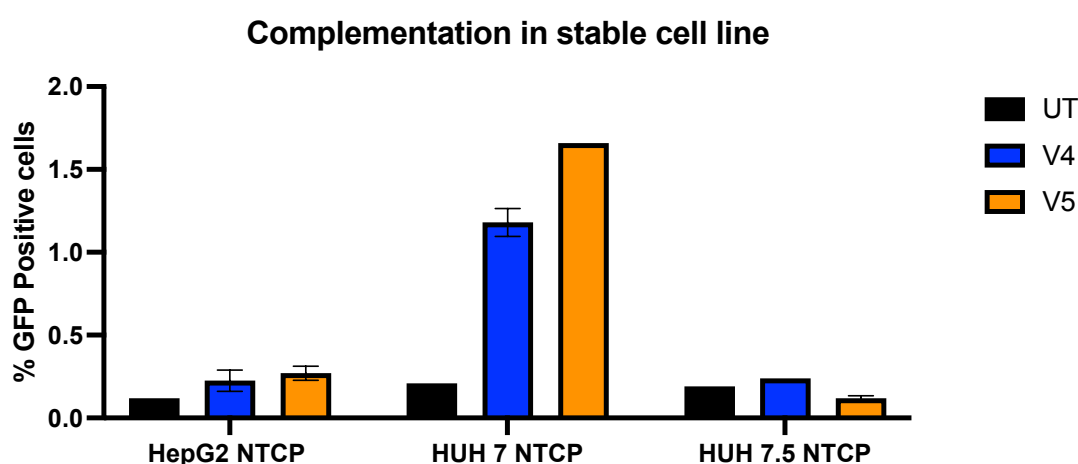


Figure 56: Evaluation of GFP complementation using stable cell lines expressing GFP1-10 SP+KDEL. Each cell line expressing GFP1-10 SP+KDEL was transfected with HBeAg V4 and V5 constructs. Results were evaluated through flow cytometry 4 days post-transfection. Data are presented as mean $\pm$ SD.

Overall, this experiment highlights the limited efficiency of the current constructs and cell systems in supporting detectable levels of GFP complementation under these conditions.

In conclusion, the reporter system is not ineffective, as we were able to detect some complementation events, even if only at low levels. However, for our purposes, we require a highly efficient reporter in which every infected cell consistently displays a detectable complementation signal. The current system does not meet this standard and is therefore not suitable for downstream applications such as CRISPR-Cas9 screening.

4.2.2 - Validation of an Anti-HBcAg Antibody

Intracellular detection of HBcAg represents a strategy to identify and quantify infected cells at the single-cell level. However, this approach requires an antibody that maintains specificity and sensitivity under intracellular staining conditions. We therefore performed a series of validation experiments to assess the performance of the selected anti-HBc antibody. To validate the antibody, we used the HepAD38 cell line, which is stably transfected with a cDNA copy of the HBV pregenomic RNA under the control of a tetracycline-inducible promoter. When cultured in the presence of tetracycline, viral replication is repressed, and thus the cells remain free of virus. Conversely, upon removal of tetracycline from the medium, cells initiate pgRNA expression, leading to active HBV replication, intracellular accumulation of viral intermediates, and secretion of virus-like particles. Under these conditions, HBV antigens, including the core protein, are consistently expressed in the entire cell population, making this system particularly suitable for antibody validation (Ladner et al. 1997).

As a control to assess the specificity of the antibody, we included a condition in which cells were treated with Myrcludex (Myr), a peptide consisting of the authentically myristoylated N-terminal 47 amino acids of the pre-S1 domain of the HBV large envelope protein. Myr specifically blocks HBV entry and thereby prevents infection (Gripon et al. 2004). Under this condition, the proportion of HBV-positive cells is expected to approach zero, as no infection should occur.

Flow cytometry analysis of HBcAg staining confirmed the specificity of the antibody employed. As shown in *Figure 57*, the negative control exhibited no detectable signal, while treatment with Myrcludex resulted in only a minimal percentage of positive cells (5%). Thereby, this result validates that the signals observed in the infected and HepAD38 samples reflect specific HBV antigen detection. Infection at 1000 mge yielded 65% HBcAg-positive cells, a result consistent with our previous observations under comparable experimental conditions. As expected, HepAD38 cells, which constitutively express HBV antigens, showed nearly complete positivity (99%), thus serving as a robust positive control.

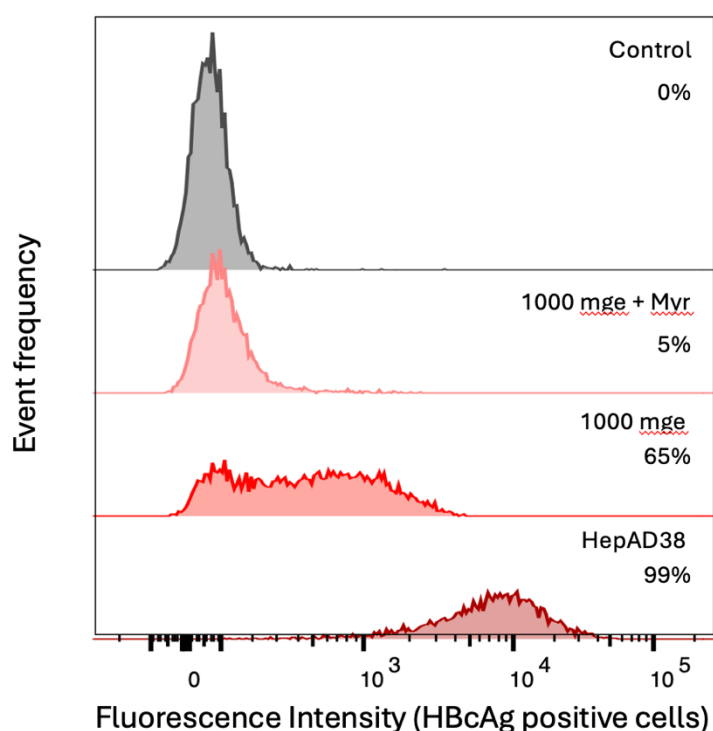


Figure 57: Flow cytometric analysis of HBcAg expression. From top to bottom: uninfected control, cells infected at 1000 mge and treated with Myrcludex, cells infected at 1000 mge, and HepAD38 cells. Six days post-infection, cells were stained with the anti-HBcAg antibody and analyzed by flow cytometry. The percentage of HBcAg-positive cells is indicated for each condition, confirming the specificity of the signal and the expected positivity profile across the experimental groups.

In conclusion, these results demonstrate that the selected anti-HBcAg antibody is both specific and sensitive in detecting intracellular HBcAg. Indeed, the expected staining patterns were observed across all experimental conditions and controls, thereby confirming the assay's reliability. This validates the antibody as a suitable tool for our screening approach, in which the ability to discriminate HBcAg-positive from negative cells at the single-cell level is essential.

5-Discussion

In this work, we addressed one of the major challenges in the study of HBV infection: the lack of a cellular model that combines robust viral permissiveness with a preserved IFN response. The identification of a model fulfilling both requirements remains particularly complex, as such conditions are rarely met simultaneously in available systems. Nevertheless, the establishment of such a model would represent a crucial advance, as it

would enable the application of CRISPR-Cas9 screening approaches to identify the mediators of the interferon-induced antiviral effect against HBV.

Importantly, HBV infection alone does not elicit a strong ISG response in the liver. However, administration of IFN- α can compensate for this lack of innate activation by establishing an antiviral state in hepatocytes through the modulation of gene expression and protein synthesis, thereby exerting non-cytolytic inhibitory effects at multiple stages of the HBV life cycle (Ye and Chen 2021). Indeed, IFN- α can block the reverse transcription of pgRNA and its export to the cytoplasm through multiple antiviral mechanisms: it induces APOBEC3 cytidine deaminases, leading to G-to-A hypermutations that inhibit HBV DNA replication (Bonvin et al. 2006); it activates MX2, which reduces viral RNA levels (Wang et al. 2020), and TRIM22, which binds the HBV core promoter to suppress transcription (Gao et al. 2009); it stimulates ISG20 and MyD88, that promote degradation of pgRNA (Liu et al. 2017), and stimulate expression of MxA that impedes HBV RNA nuclear export (Gordien et al. 2001); it alters cccDNA chromatin structure by reducing histone acetylation (H3K9, H3K27) and recruiting transcriptional corepressors such as HDAC1 and Sirt1; it decreases STAT1/STAT2 binding to cccDNA, overall leading to reduced transcription of pgRNA and subgenomic RNAs (Liu et al. 2013).

All the described mechanisms reflect the non-cytolytic antiviral functions of IFN- α observed *in vivo*, where the IFN effectively limits HBV replication through both direct actions on hepatocytes and indirect modulation of the immune response. Reproducing these effects *in vitro*, however, is challenging. Indeed, commonly used hepatoma-derived cell lines fail to adequately respond to IFN- α , making it difficult to capture the complexity of the antiviral program induced in the human liver (Caselmann et al. 1992) (Hiraga et al. 2009). Thus, identifying an immortalized human cell-based system that faithfully mirrors the *in vivo* effects of IFN- α remains a major challenge, but it is essential for advancing HBV research and for enabling the development of more physiologically relevant antiviral screening systems.

Previous studies have reported comparable limitations, suggesting that this issue is not unique but rather inherent to the field. For instance, previous studies have shown that IFN α -2b suppressed HBV replication in HepG2 cells only when applied at very high, cytotoxic concentrations. Under these conditions, the observed reduction in viral replication is likely

attributable to cell death rather than to an antiviral effect of the IFN. (Sun and Nassal 2006). Moreover, a previous study assessing the IC₅₀ of IFN- α in both PHH and HepG2-NTCP cells yielded results that are strikingly consistent with our own findings. As shown in *Table 2*, IFN- α exerts a measurable antiviral effect in primary hepatocytes, where the reduction of HBV DNA, HBsAg, and HBeAg is observed, likely due to the fact that these cells display a phenotype more closely resembling the *in vivo* condition. In contrast, for HepG2-NTCP cells, IC₅₀ values could not be accurately determined, as they exceeded the concentrations tested (Shen et al. 2018).

IC ₅₀ of IFN- α (U/mL)	PHH (9-Day Treatment)			HepG2-NTCP** (9-Day Treatment)		
	HBsAg	HBeAg	HBV DNA	HBsAg	HBeAg	HBV DNA
A2	75.0 [†]	25.5	<20	>500	>500	>500
B5	13.6	19.7	<20	>500	>500	>500
C2	11.9	11.2	<20	>500	>500	>500
D3	60.7	37.7	<20	>500	>500	>500
D	198.6 [†]	158.5*	<20	>500	>500	>500

Table 2: Comparison of IC₅₀ Concentrations (U/mL) for IFN- α Against HBV Genotype in PHH and HepG2-NTCP (Shen et al. 2018)

This finding, which is mirrored in our results, can likely be attributed to the distinct ISGs expression profiles observed in the two cell models. While the induction of primary response ISGs, such as 2'-5'-oligoadenylate synthetase 1 (OAS1), interferon-induced transmembrane protein 2 (IFITM2), STAT1, and IRF9, was similar in HepG2-NTCP cells and PHH, several ISGs previously linked to IFN- α responses in patients, including CXCL10, guanylate binding proteins (GBP5, GBP1), and tryptophanyl-tRNA synthetase (WARS), were either absent or only weakly expressed in HepG2-NTCP cells (Wu et al. 2016). In addition, key antiviral effectors such as APOBEC3G (A3G), ISG20, and APOBEC3A exhibited lower expression in HepG2-NTCP cells compared with PHH, further highlighting the more limited responsiveness of this model. In conclusion, the expression of ISGs is partially inefficient in HepG2-NTCP cells, and these differences with the PHH provide a plausible explanation for the divergent antiviral effects observed (Shen et al. 2018).

In our study, however, we identified the IFN subtype that exhibited the most potent activity against HBV across different *in vitro* experimental settings, namely IFN- α 14. In line with our findings, another study has highlighted IFN- α 14 as a particularly potent IFN- α subtype against HBV both *in vitro* and *in vivo*. Notably, IFN- α 14 was shown to elicit a coordinated activation of IFN- α and IFN- γ signaling, resulting in the robust induction of antiviral effectors and in a marked suppression of HBV transcription and antigen production (Lavoie et al. 2011). These observations not only reinforce the superior antiviral properties of IFN- α 14 compared with the conventional IFN- α 2 but also provide important mechanistic insights into its enhanced capacity to suppress HBV antigen expression and cccDNA transcription, thereby underscoring its potential for improved IFN-based therapeutic strategies.

Furthermore, the antiviral activity of IFN- α 14 could be explained by a strong and specific interaction with IFNAR1. Indeed, IFN- α 14 is the IFN- α subtype with the highest binding affinity to IFNAR1 and IFNAR2 (Lavoie et al. 2011). Consistently, an engineered IFN- α 2 mutant carrying the IFNAR1-binding sequence of IFN- α 14 (IFN- α 2–EIFK) reproduced the potent anti-HBV effects of IFN- α 14, underscoring the critical role of receptor engagement for its functional properties (Chen et al. 2021).

Supporting our results, another study demonstrated that the impaired ISGs response typically reported in HepG2-NTCP cells is largely based on analyses performed with IFN- α 2a. Indeed, treatment with IFN- α 14 was able to overcome this limitation and restore an efficient ISG signaling in this model. Specifically, IFN- α 14 was able to induce the expression of key ISGs, such as IFITM1, ISG20, GBP5, and TNFSF13B, which were absent or only weakly activated by the conventional IFN- α 2 (*Figure 58*). This restoration of ISG responsiveness was accompanied by markedly enhanced antiviral efficacy. Collectively, these findings highlight that the magnitude and quality of ISG induction play a pivotal role in shaping the cellular response to IFN therapy, and that IFN- α 14, by restoring this ISG phenotype, emerges as a particularly promising candidate for HBV control (Shen et al. 2018).

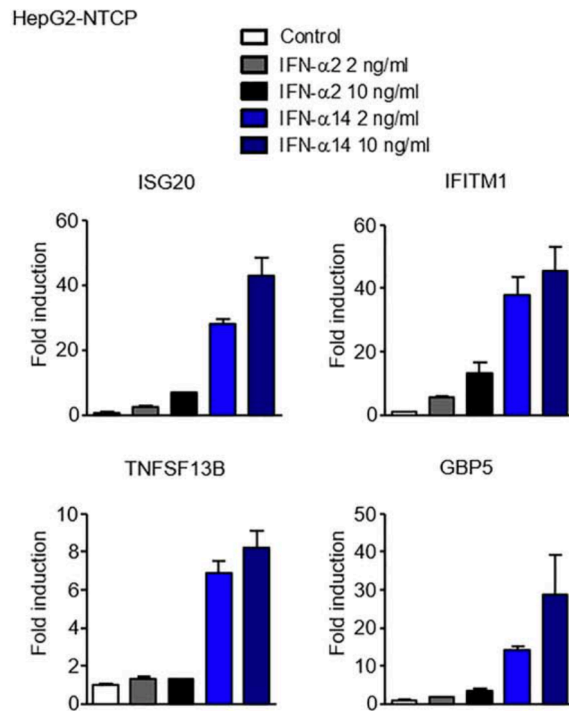


Figure 58: IFN- α 14- or IFN- α 2-induced expression of ISGs (Shen et al. 2018)

The evaluation of different cellular models highlighted that the HepG2-NTCP line, when cultured in the presence of 2.5% DMSO, represents the optimal balance between infection efficiency and sensitivity to interferon response. Indeed, our data enabled the identification of an experimental condition ideally suited for a CRISPR-Cas9–based genomic screening. This finding is consistent with previous observations attributing to DMSO a role in promoting hepatocytic differentiation and stabilizing cellular functions associated with liver metabolism.

Although primary human hepatocyte cultures remain the gold standard, their practical disadvantages, such as restricted availability, inability to proliferate, and substantial donor-to-donor variability, limit their utility. For these reasons, alternative hepatocyte models have been explored, particularly hepatoma-derived cell lines such as HepG2. Previous studies have shown that DMSO treatment can promote differentiation and enhance drug metabolism in these cell lines, although the underlying molecular mechanisms are not fully understood (Choi et al. 2009). Among the reported effects of DMSO, there are: decreased proliferation rates and induction of a more differentiated phenotype (Sainz and Chisari 2006). Supporting this thesis, another study detected DMSO as a reducer of the malignant phenotype in HepG2, demonstrated by a significant decrease in the tumor marker alpha-fetoprotein (AFP) mRNA

expression, together with an enhancement of hepatocyte-like metabolic functionality. These findings underscore the potential of DMSO-treated hepatoma-derived models to mimic primary hepatocytes more closely, thereby providing a system that better recapitulates the physiological features of PHH (Nikolaou et al. 2016).

However, DMSO treatment may not represent a universal solution across all hepatoma-derived cell lines, even though it appears effective for HepG2 cells. For instance, Huh 7.5-NTCP cells cultured with 2% DMSO for one week were shown to undergo partial hepatocytic differentiation (dHuh 7.5-NTCP). Yet, despite this conditioning, subsequent treatment with IFN- α failed to reduce HBV RNA levels, indicating that the cells remained unresponsive to interferon (Fouillé et al. 2025). This underscores that the identification of a continuous hepatoma-derived cell line that is both permissive to HBV infection and responsive to physiologically relevant concentrations of interferon remains a significant challenge.

Nevertheless, our study provides an important contribution by demonstrating that, using HepG2-NTCP seeded in 2,5% DMSO, the response to IFN- α 14 is not only preserved but also functionally robust, thereby validating this platform as suitable for large-scale functional screening. Importantly, our study reveals a clear distinction between different interferon subtypes in this system. While IFN- α 14 alone exerts a stronger antiviral effect than IFN- α 2a, its combination with DMSO produces a markedly enhanced response that closely recapitulates the antiviral activity observed in PHH. By contrast, IFN- α 2a remains ineffective even when combined with DMSO, indicating that the potentiation we observed is not a universal feature of all IFNs. Therefore, the robust antiviral outcome seen with IFN- α 14 arises from the convergence of two complementary phenotypic improvements: DMSO-induced hepatocytic differentiation and the ability of IFN- α 14 to restore the induction of ISGs essential for antiviral defense. Together, these findings underscore that both elements are required to elicit a physiologically relevant antiviral interferon response in HepG2-NTCP cells, highlighting IFN- α 14 as uniquely capable of driving this effect.

In addition, we sought to develop an HBV reporter virus to facilitate screening strategies by enabling the direct discrimination between HBV-positive and HBV-negative cells. A critical aspect of this work was therefore the establishment of a robust and efficient method to identify HBV-infected cells. Although reporter systems based on recombinant HBV viruses

have proven useful in other virological contexts (Sumiyadorj et al. 2022), their applicability in our model was limited, as the system's design does not permit single-cell analysis but only provides an overall readout based on luciferase. In fact, the ability to distinguish between infected and uninfected cells is crucial for conducting a CRISPR-Cas9 screening, where cells with specific knockouts must be isolated and subsequently analyzed.

The development of an HBV reporter virus is hindered by the compact and highly overlapping organization of its genome. This arrangement restricts the insertion of exogenous sequences, as even small genetic modifications can compromise viral replication, infectivity, or genome stability. Indeed, to establish our HBV reporter system, we employed the self-complementing split-GFP approach, which relies on the reconstitution of fluorescence from two engineered fragments: the small peptide tag GFP11, corresponding to the 11th β -strand, and the non-fluorescent detector fragment GFP1–10, comprising β -strands 1–10. A key requirement in this design is the selection of a viral protein that is dispensable for replication and that still tolerates the insertion of a minimal tag. We therefore focused on HBeAg, which is not required for viral replication but widely used as a marker of HBV replication activity. Although the mature protein is secreted, the existence of intracellular intermediates suggested that fusion with GFP11 could complement cytosol-retained GFP1–10, thereby restoring fluorescence.

Despite our attempts to enhance the reporter signal by engineering the cytosolic portion of GFP1–10 to increase the likelihood of interaction with HBeAg-linked GFP11, the overall complementation efficiency remained low, with detectable fluorescence observed only in a minority of transfected cells. These results indicate that GFP reconstitution occurs sporadically, reflecting both the limited capacity of the current constructs and potential restriction imposed by HBV genome organization. While complementation events confirm the plausibility of the approach, the system lacks the robustness required for a reliable reporter, as efficient signal detection in all infected cells is essential for its intended application.

As an alternative, we optimized an immunostaining protocol for HBeAg, which provided superior sensitivity and reproducibility. This methodological advance represents a powerful tool not only for large-scale genetic screening but also for fundamental studies of the HBV life cycle.

The model described in this work offers a robust platform for elucidating the biology of interferons and their antiviral mechanisms. Notably, our data demonstrate that the combination of DMSO-induced differentiation with IFN- α 14 stimulation is essential to achieve a physiologically relevant *in vitro* system in which a clear antiviral effect against HBV can be observed. This unique synergy makes HepG2-NTCP cells a reliable platform not only for mechanistic studies but also for large-scale functional applications. In particular, the integration of this system with CRISPR-Cas9 libraries targeting ISGs will enable the systematic delineation of the molecular landscape underlying antiviral responses against HBV, thereby identifying the effector of IFN. Furthermore, the ability to directly compare the activity of distinct interferon subtypes within the same cellular context provides valuable insights into their differential antiviral potential *in vitro*. Overall, this work has defined a stable, sensitive, and biologically relevant system for the study of HBV infection and interferon responses. Indeed, the findings presented here not only establish the prerequisites for genome-wide screening studies but also clarify some poorly understood aspects of HBV-interferon interactions. The approach developed thus represents a significant step forward in understanding the mechanisms of viral control and in guiding the design of innovative therapeutic strategies against CHB.

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7 – Activities during the PhD

7.1- List of Publications

1: Brasu N, Elia I, Russo V, Montacchiesi G, Stabile SA, De Intinis C, Fesi F, Gizzi K, Macagno M, Montone M, Mussolin B, Grifoni A, Faravelli S, **Marchese S**, Forneris F, De Francesco R, Sette A, Barnaba V, Sottile A, Sapino A, Pace L. Memory CD8⁺ T cell diversity and B cell responses correlate with protection against SARS-CoV-2 following

mRNA vaccination. *Nat Immunol.* 2022 Oct;23(10):1445-1456. doi: 10.1038/s41590-022-01313-z. Epub 2022 Sep 22. PMID: 36138186.

2: D'Acunto, Emanuela, Alessia Muzi, **Silvia Marchese**, Lorena Donnici, Valerio Chiarini, Federica Bucci, Emiliano Pavoni, Fabiana Fosca Ferrara, Manuela Cappelletti, Roberto Arriga, and et al. 2024. "Isolation and Characterization of Neutralizing Monoclonal Antibodies from a Large Panel of Murine Antibodies against RBD of the SARS-CoV-2 Spike Protein" *Antibodies* 13, no. 1: 5. <https://doi.org/10.3390/antib13010005>

3: Andreano E, Paciello I, Pierleoni G, Piccini G, Abbiento V, Antonelli G, Pileri P, Manganaro N, Pantano E, Maccari G, **Marchese S**, Donnici L, Benincasa L, Giglioli G, Leonardi M, De Santi C, Fabbiani M, Rancan I, Tumbarello M, Montagnani F, Sala C, Medini D, De Francesco R, Montomoli E, Rappuoli R. B cell analyses after SARS-CoV-2 mRNA third vaccination reveals a hybrid immunity like antibody response. *Nat Commun.* 2023 Jan 4;14(1):53. doi: 10.1038/s41467-022-35781-6. PMID: 36599850; PMCID: PMC9811867.

4: Fumagalli V, Ravà M, Marotta D, Di Lucia P, Bono EB, Giustini L, De Leo F, Casalgrandi M, Monteleone E, Mouro V, Malpighi C, Perucchini C, Grillo M, De Palma S, Donnici L, **Marchese S**, Conti M, Muramatsu H, Perlman S, Pardi N, Kuka M, De Francesco R, Bianchi ME, Guidotti LG, Iannaccone M. Antibody-independent protection against heterologous SARS-CoV-2 challenge conferred by prior infection or vaccination. *Nat Immunol.* 2024 Apr;25(4):633-643. doi: 10.1038/s41590-024-01787-z. Epub 2024 Mar 14. PMID: 38486021; PMCID: PMC11003867.

5: Andrea Favalli, Giorgio Patelli, Paola Gruarin, Andrea Gobbini, Elisa Pesce, Sara Mariano, Mauro Bombaci, Francesca Vincenti, Lorena Donnici, **Silvia Marchese**, Daniele Piscazzi, Alessio Amatu, Federica Tosi, Silvia Ghezzi, Arianna Pani, Silvia Principato, Andrea Lombardi, Alessandra Bandera, Sergio Abrignani, Salvatore Siena, Andrea Sartore-Bianchi and Renata Maria Grifantini. Impact of chemotherapy on humoral and cellular immune responses to COVID-19 vaccination in patients with solid tumors. *Frontiers in Immunology*, section Immunological Memory. Volume 16 - 2025 | <https://doi.org/10.3389/fimmu.2025.1664072>

7.2- List of Congress

(25/06/2023-27/06/2023) – 7th National Congress of the Italian Society of Virology. One Virology One Health. Brescia, Italy.

(16/02/2023-18/02/2023) – 1st Retreat of the Department of Pharmacological and Biomolecular Sciences “Rodolfo Paoletti” – “TO EXCELLENCE AND BEYOND”, Riccione, Italy

7.3- Tutor Activities

(04/06/2024-12/06/2024) 32 hours of laboratory assistance activities in the course of “Microbiology and Hygiene” under the supervision of Dr. Lara Manganaro.

7.4- Study Abroad Experience

(01/03/2025-05/06/2025) - Study abroad period at The Westmead Institute for Medical Research (WIMR) in the laboratory of A/Prof Thomas Tu, where I deepened my research on hepatitis B.