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Hepatitis B Virus X Protein: A Key Regulator of the Virus Life Cycle

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1. Introduction

Hepatitis B virus (HBV) is one of the most important human pathogens. The outcome of HBV infection as well as the severity of HBV-induced liver disease varies widely from one patient to another. In around 90-95% of adults, exposure to HBV leads to an acute infection which is rapidly cleared without long-term consequences. The remaining 5-10% fail to control viral infection that consequently evolves to chronicity. The rate of chronicity of viral infection is dramatically higher (up to 90%) in neonates born from infected mothers, suggesting that infection around birth successfully induces peripheral tolerance to viral antigens which prevents clearance. About 2 billion humans have been infected by HBV worldwide and more than 350 million are chronic carriers. The latter have high risk to develop severe liver disease, including liver cirrhosis and hepatocellular carcinoma. Around 600,000 persons die each year due to consequences of hepatitis B infection. As HBV is a non-cytopatic virus, HBV-related liver damage very likely results from the immune response against infected hepatocytes which is activated but not strong enough to clear infection.

Our knowledge of the molecular biology of HBV has increased considerably over the past decades, leading to the development of very effective prophylactic vaccines and to the development of direct antivirals active against HBV. Five nucleos(t)ide analogs are currently approved to treat chronic hepatitis B. Belonging to the same class of nucleosidic reverse transcriptase inhibitors, they specifically inhibit viral polymerase activity and thus suppress HBV replication, significantly improving liver histology and the clinical outcomes of the disease after one year of treatment (Liaw *et al.*, 2004). Unfortunately, nucleos(t)ide analogs act at a late stage in the HBV life cycle (i.e. maturation of newly formed viral capsids by reverse transcription of pregenomic RNA) and do neither prevent formation and nuclear establishment nor activity of the HBV transcription template, the so called HBV covalently closed circular (ccc) DNA.

Long-term treatments with nucleos(t)ide analogs are thus necessary to cure HBV infected cells and unfortunately lead to the selection of HBV drug-resistant strains (Zoulim, 2006). Even very effective antivirals such as Tenofovir lead to HBsAg seroconversion in only 3 to 8% of patients over three years (Heathcote *et al.*, 2011; van Bommel *et al.*, 2010). Pegylated (PEG)-IFN-α is an established treatment alternative and acts as an antiviral but also enhances the host's immune defense. However, only 30% of PEG-IFN-α -treated patients

achieve a sustained antiviral response (Karayiannis, 2003), and only about 8-10 % of patients clear the virus (Marcellin *et al*., 2009) with slightly increasing rates during long-term follow-up (Moucari *et al*., 2009). New therapeutic approaches that target other viral proteins, besides viral polymerase, are needed to decrease viral drug resistance and improve treatments against HBV.

This chapter will particularly focus on the hepatitis B virus X protein (HBx) that is essential to initiate and maintain transcription of HBV RNA from nuclear cccDNA and thus is a key regulator of the virus life cycle. Due to its central role, HBx represents a very promising new target for antiviral strategies against HBV.

1.1 Hepatitis B virus structure and proteins

HBV belongs to the family *hepadnaviridae*. It is a small, enveloped DNA virus that replicates via reverse transcription of an RNA intermediate. HBV virions, also called Dane particles, are spherical lipid-containing structures with a diameter of ~42 nm (Fig. 1). The inner shell

A

HBV proteins	
S	small surface protein
M	middle surface protein
L	large surface protein
core	capsid protein
HBeAg	secreted e antigen
pol	polymerase
HBx	X protein (non-secreted)

B

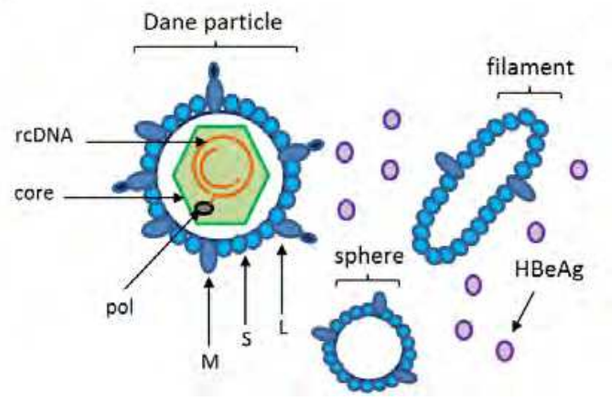

Fig. 1. HBV proteins and virion structure. (A) List of all HBV proteins. (B) Viral particles present in the serum of HBV-infected patients are schematically represented. The so-called "Dane particles" are fully infectious viral particles containing the HBV capsid and one rcDNA genome copy with the viral polymerase attached. Subviral particles of spherical or filamentous shapes consist of empty viral envelopes. Together, Dane particles, spheres, and filaments are recognized as HBsAg. The precore protein is secreted as HBeAg.

of the virus consists of an icosahedral capsid, which is assembled from 180 or 240 subunits of the core protein. The capsid is covered by a lipid bilayer membrane densly packed with the three envelope proteins, large (L), middle (M), and predominantly small (S) protein, and is acquired by budding into the endoplasmic reticulum. They are translated from individual start codons but share the open reading frame and the same C-terminal amino acids, called the S domain. As a consequence, the M protein shares the S and has an extra N-terminal domain called preS2, and the L protein encompasses the S and two extra domains: preS2 and preS1. Capsids contain a single copy of the HBV genome consisting of a 3.2-kb partially double-stranded relaxed circular (rc) DNA molecule. The viral polymerase serves as a protein primer and remains covalently linked to the 5′ end of the complete strand, also called viral (–) strand DNA of the rcDNA after reverse transcription. Besides virions, HBV infection leads to secretion of huge amounts of subviral particles, which consist of empty

viral envelopes with filamentous or spherical shapes (Fig. 1) containing mainly S and little L protein. Subviral particles are the most abundant HBV structures released into the bloodstream, are commonly defined as hepatitis B surface (HBs) antigen and are thought to facilitate virus spread and persistence in the host by adsorbing virus-neutralizing antibodies and tolerizing T cell responses.

In addition to polymerase and the structural proteins, the HBV genome also encodes for two non-structural proteins, which have less well-defined functions. Secreted HBeAg may have immunoregulatory functions (Bertoletti & Gehring, 2006; Chen *et al*., 2005; Chen *et al*., 2004; Visvanathan *et al*., 2007), whereas HBx seems to have multiple key functions as it will be detailed later.

1.2 Overview of the hepatitis B virus life cycle

A schematic overview of the HBV life cycle is depicted below in Fig. 2. HBV infection is restricted to hepatocytes. HBV entry into these cells is thought to be a multistep process. Virions are first trapped at the surface of the cell by heparan sulfate proteoglycans (Schulze *et al*., 2007) and then bind to a receptor allowing uptake into the cells via an endocytosis process (Kott, 2010; Leistner *et al*., 2008). So far, this cellular receptor as not been identified. Proteolytic cleavage of the surface protein occurs within the endosomal compartment, probably resulting in a conformational change that exposes some translocation motifs at the surface of the viral particle allowing fusion of viral and cellular membranes and release of the capsid into the cytosol (Stoeckl *et al*., 2006).

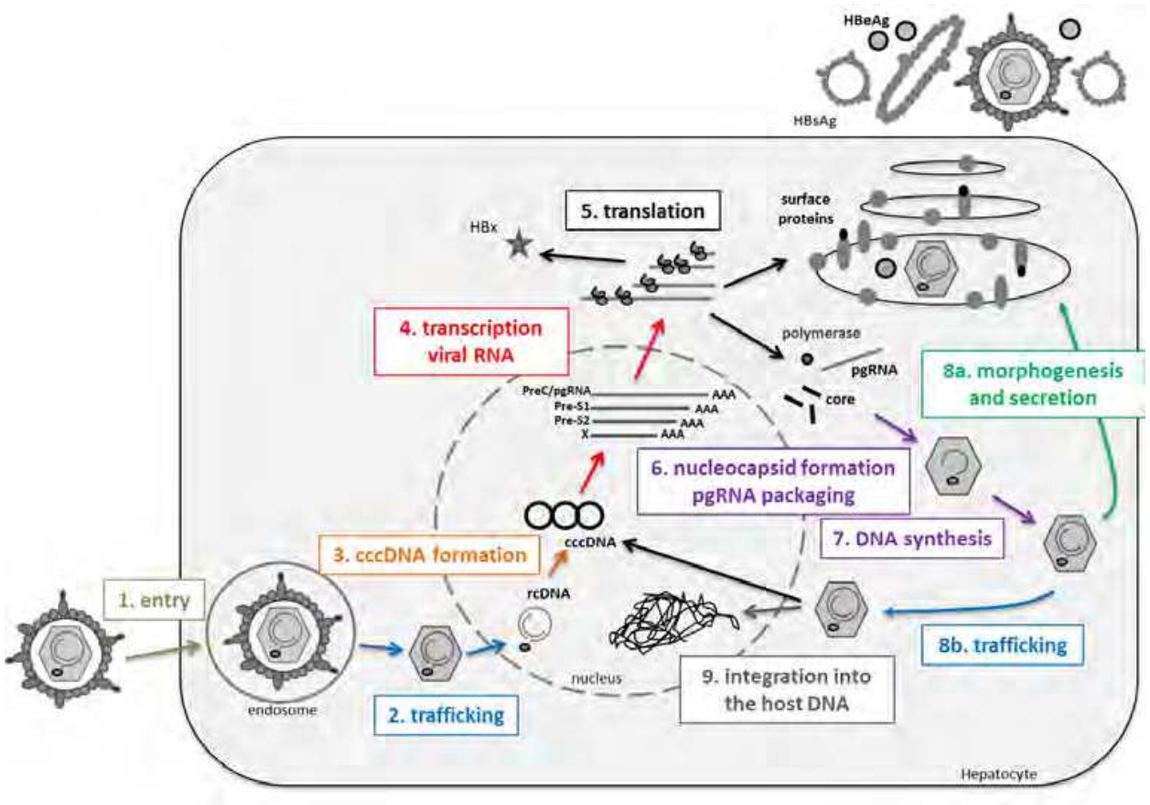

Fig. 2. Schematic overview of the HBV life cycle.

The naked capsid is then directed towards the nucleus, and the HBV genome is translocated to the nucleus (Rabe *et al.*, 2006). In the nucleus, the rcDNA genome is converted by cellular enzymes into a covalently closed circular DNA (cccDNA), the episomal persistance form of the virus serving as transcription template. The 3.5 kb RNA species serves as pregenomic RNA (pgRNA) and as messenger RNAs for the synthesis of polymerase and core proteins as well as HBeAg. The 2.1 and 2.4 kb subgenomic RNAs encode for the three viral envelope proteins, a small 0.7 kbRNA for the HBx. The pgRNA is exported in an unspliced form, encapisidated together with the viral polymerase and used as a template for reverse transcription. The capsid spontaneously self-assembles from core dimers present in the cytoplasm (Zlotnick *et al.*, 1999) due to the nucleic acid-binding domain of the core protein. Specific packaging of pgRNA into the capsid is mediated by binding of the primer region of the viral polymerase to the ε stem-loop in the 5′ region of pgRNA (Hirsch *et al.*, 1990; Junker-Niepmann *et al.*, 1990; Knaus & Nassal, 1993; Nassal, 1992; Porterfield *et al.*, 2010). The pgRNA is then reverse transcribed by the reverse transcriptase domain of the polymerase within the capsid in the cytoplasm of the infected cell. Upon minus and then plus strand DNA synthesis the capsid matures and can be enveloped or reimported into the nucleus to fill up a cccDNA pool.

HBV budding has been shown to be strictly dependent on the L protein (Bruss & Vieluf, 1995): when the ratio between L proteins and nucleocapsids is not optimal, the latter are preferentially targeted to the nucleus to amplify the cccDNA pool (Summers *et al.*, 1990). Whether HBV virions bud into the endoplasmic reticulum or late endosomes or multivesicular bodies, before they exit the cell via the exosome pathway, is not entirely clear (Patient *et al.*, 2009). As an alternative and although it is not essential for the HBV life cycle, the viral genome may also integrate into the host genome using cellular enzymes such as topoisomerase I (Wang & Rogler, 1991).

1.3 General features about HBx

HBx is translated from a small subgenomic RNA controlled by the HBx promoter (Guo *et al.*, 1991). Alternatively, HBx may be produced form a very long RNA (3.9 kb) containing all the HBV open reading frames (ORF) (Doitsh & Shaul, 2003). The ORF was originally designated X because of the lack of homology with known sequences. HBx is a protein composed of 154 amino acid residues with a molecular mass of around 17.5 kDa. Due to the lack of successful crystallography analyses, little is known about its three dimensional structure. Post-transcriptional modifications of HBx such as phosphorylation or acetylation have been described (Schek *et al.*, 1991; Urban *et al.*, 1997), the latest being observed only in insect cells. But the significance of such modifications for the described activities of HBx has not been assessed yet.

Cellular localization of HBx has been debated over the years. Indeed, some studies show a cytoplasmic localization (Dandri *et al.*, 1996; Doria *et al.*, 1995; Sirma *et al.*, 1998; Su *et al.*, 1998), whereas others find that HBx is preferentially nuclear (Weil *et al.*, 1999), or present both in the cytoplasm and the nucleus (Hoare *et al.*, 2001; Schek *et al.*, 1991). It appears that HBx expressed at very low level is predominantly nuclear, whereas high levels of HBx lead to cytoplasmic accumulation (Cha *et al.*, 2009; Henkler *et al.*, 2001). Discrepancies regarding HBx localization could thus be attributed to variations of HBx expression levels according to the models used for the experiments in the different studies.

Cellular localization of HBx was shown to influence the half-life of the protein. Indeed, the pool of HBx associated with the cytoskeleton and nuclear framework has a longer half-life (around 3 h) than the one associated with the cytosolic fraction (15 to 20 min) (Dandri *et al.*, 1998; Schek *et al.*, 1991). Both ubiquitin-dependent and ubiquitin-independent mechanisms have been involved in HBx turnover (Hu *et al.*, 1999; Kim *et al.*, 2008).

2. Importance of HBx for HBV infection

In the woodchuck model of HBV infection, it was shown that the woodchuck hepatitis virus (WHV) X protein (WHx) is essential for the establishment of viral infection *in vivo* (Chen *et al.*, 1993; Zoulim *et al.*, 1994). Indeed, injection of WHV wild type genomes into the liver of woodchuck lead to WHV infection of all the tested animals whereas no replication was observed when genomes deficient for WHx expression were injected (Chen *et al.*, 1993; Zoulim *et al.*, 1994). Few years later, it was observed that animals injected with WHx-defective mutants eventually developed a low viremia after an extended period of time (Zhang *et al.*, 2001), suggesting that this WHx-defective mutant were not completely defective but largely attenuated for HBV replication *in vivo*. Accordingly, genotypic reversions to wild type WHV were observed in all animals inoculated with WHx-deficient mutants (Zhang *et al.*, 2001). Taken together, these results point out the importance of WHx for a productive and long lasting WHV infection.

In addition, it was shown that HBx-deficient HBV genomes are somewhat compromised for HBV replication using HBV hydrodynamically-injected mice (Keasler *et al.*, 2007; 2009) or cell culture models (Belloni *et al.*, 2009; Blum *et al.*, 1992; Keasler *et al.*, 2007; Leupin *et al.*, 2005). Surprisingly, the absence of HBx had no effect on HBV replication in human hepatoma Huh7 cell lines, but impaired replication in HepG2 cells (Blum *et al.*, 1992; Keasler *et al.*, 2007; 2009; Leupin *et al.*, 2005). Accordingly, data in HBV transgenic mice are contradictory with some mouse lines showing reduced replication (Xu *et al.*, 2002), whereas others replicate HBV to high levels (Dumortier *et al.*, 2005).

The importance of HBx in the context of human HBV infection was demonstrated very recently using human hepatocyte chimeric mice and relevant cellular models of HBV infection. Indeed, it was observed that mice injected with HBx deficient HBV virus developed measurable viremia only in HBx-expressing livers (Tsuge *et al.*, 2010). Moreover, using primary human hepatocyte (Schulze-Bergkamen *et al.*, 2003) and differentiated HepaRG cells (Gripon *et al.*, 2002), that are the only two models of HBV infection *in vitro*, we recently demonstrated that HBx is essential to initiate and constantly required to maintain productive HBV infection (Lucifora *et al.*, 2011).

This latter study highlighted the importance of performing experiments in relevant *in vitro* and *in vivo* models. Indeed, results obtained with *in vitro* HBV infection models (i.e. primary human hepatocyte and differentiated HepaRG cells) (Lucifora *et al.*, 2011) support and explain the above mentioned observations obtained in mouse livers (Keasler *et al.*, 2007; Tsuge *et al.*). However, they differ from results obtained by transfection of linearized HBV genomes into transformed cells (Blum *et al.*, 1992; Leupin *et al.*, 2005) especially when HBx is overexpressed to non-physiological levels. Solving this apparent discrepancy, we were able to demonstrate that HBx is essential when HBV transcription is initiated from its natural transcription circular template (cccDNA) but not from a linearized 1.3-fold genome length

HBV genome (Lucifora *et al.*, 2011) containing a duplicate copy of the HBx open reading frame 5′ of the HBV genome (Reifenberg *et al.*, 2002; Sprinzl *et al.*, 2001; Zhang *et al.*, 2004) – irrespective of whether the linearized HBV genome is integrated or episomal.

3. Functions of HBx in the HBV life cycle

Different functions have been attributed to HBx regarding HBV life cycle (Fig. 3).

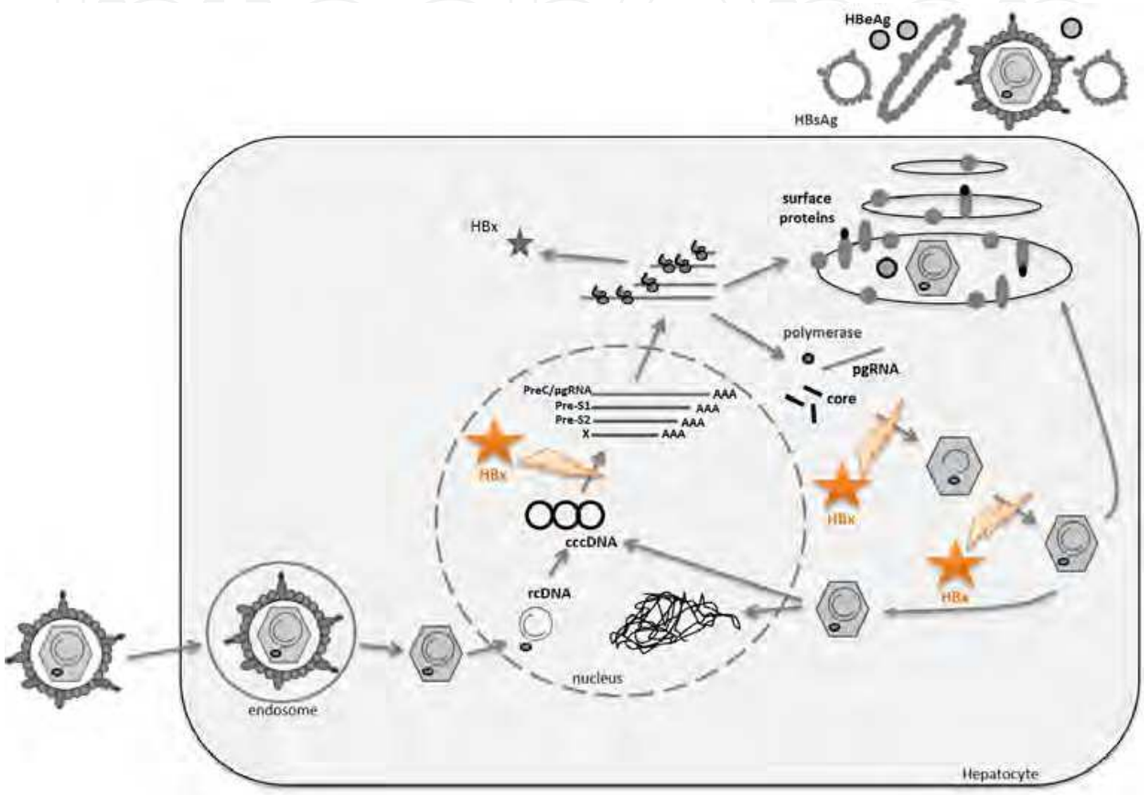

Fig. 3. Functions attributed to HBx in the HBV life cycle. HBx is an important regulator of HBV transcription. Moreover, it might also enhance pgRNA encapsidation and viral polymerase activity.

Several studies have shown that HBx can stimulate HBV replication by activating viral transcription (Cha *et al.*, 2009; Leupin *et al.*, 2005; Tang *et al.*, 2005; Zhang *et al.*, 2004; Zhang *et al.*, 2001) or enhancing viral polymerase activity via calcium signalling pathways (Bouchard *et al.*, 2003; Bouchard *et al.*, 2001; Klein *et al.*, 1999). HBx was also proposed to enhance pgRNA encapsidation by increasing phosphorylation of the viral core protein (Melegari *et al.*, 2005) although these results were recently challenged (Cha *et al.*, 2009).

We recently showed that HBx does not determine the ability of HBV to enter the host cell or to deposit functional nuclear cccDNA but is essential for viral transcription from its natural transcription template, the nuclear HBV cccDNA (Lucifora *et al.*, 2011). Indeed primary human hepatocytes or differentiated HepaRG cells inoculated with different HBV virions, HBV(wt) and HBV(x-) established comparable amounts of nuclear transcription templates but in contrast to HBV(wt), transcription of HBV RNAs and expression of HBV proteins was

dramatically impaired in cells inoculated with HBV(x-) (Lucifora *et al.*, 2011). Trans-complementation of HBx in HBV(x-)-infected cells was able to rescue HBV transcription, antigen secretion and replication even weeks after infection. This demonstrated that HBx-deficient cccDNA is fully functional and very stable, but also that HBx is necessary to initiate and maintain HBV replication after infection of human hepatocytes (Lucifora *et al.*, 2011).

Our results complement a series of data indicating that HBx has an important role in epigenetic regulation of HBV transcription from cccDNA. Indeed, cccDNA can persist in the cell nucleus as a stable chromatin-like episome (Bock *et al.*, 2001) and was shown to be submitted to epigenetic modifications such H3 and H4 histone acetylations when HBV was actively replicating (Pollicino *et al.*, 2006). Besides cellular proteins such as histone acetyltransferases and histone deacetylases, HBx is also recruited onto the cccDNA with a kinetic paralleling HBV replication (Belloni *et al.*, 2009). Moreover, in the absence of HBx, the acetylation of cccDNA-bound histones H4 was significantly reduced (Belloni *et al.*, 2009; Lucifora *et al.*, 2011), the recruitment of the histone acetyltransferase p300 was severely impaired whereas the recruitment of the histone deacetylases hSirt1 and HDAC1 was increased and occured at earlier times (Belloni *et al.*, 2009).

The differences mentioned above in the regulation of viral transcription from cccDNA and from linearized HBV genomes (which are present in all plasmid constructs and stable cell lines) may help to explain, why the function of HBx was evaluated differently when different HBV constructs were used (Blum *et al.*, 1992; Bouchard *et al.*, 2001; Melegari *et al.*, 2005; Reifenberg *et al.*, 2002; Sprinzl *et al.*, 2001). However, transcriptional regulation by HBx may also depend on the cell type used, since transformed cells may lack or antagonize cellular proteins with a positive or negative influence on viral transcription.

Although HBx is essential for the expression of the other viral proteins, no evidence for packaging of HBx into the HBV particle has been provided (Lucifora *et al.*, 2011). Therefore, the question of how HBx expression itself is induced and regulated remains open. Different hypotheses may apply. First, HBx mRNA transcription may be specifically regulated and may occur before transcription of the other HBV RNAs. This implies the question whether an early-late shift exists for HBV such as for most other viruses – with HBx as an early protein essential for expression of the remaining (late) proteins. Some studies performed in transfection models support this assumption (Doitsh & Shaul, 2004; Wu *et al.*, 1991) suggesting that HBV may express its gene products in a defined order.

A second hypothesis does not require the presence of HBx in the early phase of HBV infection. If HBV transcription from cccDNA starts shortly after infection independent from HBx, this would lead to the production of all the HBV proteins including HBx. Subsequent activation of a cellular response controlling HBV replication and/or binding of cellular restriction factor(s) could - in the absence of HBx - inhibit HBV transcription from cccDNA. HBx would here be essential to prevent inhibition of HBV transcription by cell-intrinsic mechanisms. Since HBx would have to up-regulate its own expression in a "positive feed-back loop", this would explain why a lag phase is observed before HBV replication starts after infection in all the HBV infection models (Dandri *et al.*, 2005; Gripon *et al.*, 1988; Gripon *et al.*, 2002; Walter *et al.*, 1996; Wieland *et al.*, 2004). Whether one of these hypotheses or a third one explains dependency of HBV replication on HBx is currently investigated.

4. HBx influences many cellular processes

Besides its role in HBV replication, thousands of publications showed that HBx interacts with various cellular partners and modifies many cellular processes including transcription, cell cycle progression, DNA damage repair, apoptosis and carcinogenesis (for review, see Benhenda *et al.*, 2009; Bouchard & Schneider, 2004; Wei *et al.*, 2010). As we will show with the following examples, interactions of HBx with cellular components may represent an attempt of the virus to manipulate the cellular context in order to stimulate virus replication and spread.

HBx has been described to be a weak transactivator able to activate HBV promoters and enhancers as well as many different cellular promoters (Yen, 1996). Whereas, HBx does not seem to directly bind to DNA, its transactivation activity was reported to occur via several DNA binding sites such as NF-KB, AP-1, c-EBP, ATF/CREB, NF-AT, SP1 etc. (for review, see Quasdorff & Protzer, 2010; Yen, 1996).

Different studies have shown an interplay between HBx and apoptosis pathways. Indeed, HBx could sensitize the cells to apoptotic signals such as treatments with TNF or doxorubicin, oxidative stress or growth factor deprivation (for review, see Benhenda *et al.*, 2009; Bouchard & Schneider, 2004; Wei *et al.*, 2010). This may promote hepatocyte regeneration, thus providing a larger reservoir of cells for infection. However HBx may also prevent apoptosis induction since it rapidly blocks spread of HBV progeny (Arzberger *et al.*, 2010).

HBx may also be involved in cell cycle regulation but its relative influence seems to differ according to the models used (for review, see Benhenda *et al.*, 2009; Bouchard & Schneider, 2004; Wei *et al.*, 2010). For example, using primary rat hepatocytes, it was recently demonstrated that HBx induces normally quiescent hepatocytes to enter the G_1 phase of the cell cycle and that this calcium-dependent HBx activity is required for HBV replication (Gearhart & Bouchard, 2010). While this effect of HBx on cell cycle progression can probably lead to carcinogenesis and thus become deleterious for the host, it is believed that it might be important for the virus to induce expansion of available deoxynucleoside triphosphate pools within the cells which it needs for replication (Bouchard *et al.*, 2003). Indeed, using HepG2 cells, it was reported that HBx is sufficient for the induction of the R2 subunit of the ribonucleotide reductase (RNR) (Cohen *et al.*, 2010). RNR is the key enzyme responsible for *de novo* dNTP synthesis and is composed of R1 and R2 subunits (Nordlund & Reichard, 2006). While the R1 subunit is expressed in quiescent cells, the R2 subunit expression is silenced (Chabes *et al.*, 2003). As a consequence of induction of R2 by HBx, the dNTP pool for effective viral production was increased without affecting cell cycle progression (Cohen *et al.*, 2010).

Different groups using different models showed that HBx may localize and interact with the proteasome components thereby influencing proteasome subunit composition (Chen *et al.*, 2001; Fischer *et al.*, 1995; Hu *et al.*, 1999; Zhang *et al.*, 2000). Moreover proteasome inhibition was shown to enhance HBV replication in cell culture and in mice models (Zhang *et al.*, 2004; Zhang *et al.*, 2010). Indeed, in the presence of proteasome inhibitors, the replication of the wild-type virus was not affected, while the replication of the HBx-negative virus was enhanced and restored to the wild-type level (Zhang *et al.*, 2004; Zhang *et al.*, 2010). Thus HBx may functions through the inhibition of proteasome activities to enhance HBV replication.

Finally, several studies have pointed out an interaction between HBx and the DNA repair protein DDB1 that would be essential for HBV infection (Leupin *et al.*, 2005; Sitterlin *et al.*, 2000). However, the exact mechanism by which this interaction may help the virus is still debated.

Of note, most of the interactions of HBx with cellular processes have been studied in many different models often leading to significant overexpression of HBx and outside the context of HBV infection. Thus, it remains important to determine whether similar manipulations of the cellular machinery by HBx would also occur in the context of an authentic HBV infection.

5. Conclusion

Numerous and significant studies have been performed over the past decades to analyze the role of HBx in the HBV life cycle. Many data were generated by using different *in vivo* and *in vitro* models, but contradictory results describing HBx function were obtained. The importance and the precise role of HBx on HBV life cycle thus remained unclear until recently models allowing an authentic HBV infection were used (Lucifora *et al.*, 2011; Tsuge *et al.*, 2010). Most studies, including the most recent, agree that HBx is essential for HBV infection. Besides its importance for HBV transcription from nuclear HBV cccDNA, it may also influence downstream steps of the HBV life cycle possibly by manipulating different cellular machineries. Unfortunately, in the long-term, these manipulations are probably leading to hepatocellular de-differentiation and progression towards liver cancer. As HBx plays a central role in HBV infection and cannot avoid influencing many cellular processes related to disease progression, it may be a very interesting target for new therapies against chronic hepatitis B. Targeting HBx may prevent both: viral replication as well as liver tissue damage and carcinogenesis.

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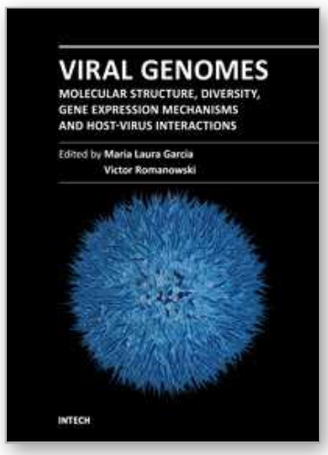

Viral Genomes - Molecular Structure, Diversity, Gene Expression Mechanisms and Host-Virus Interactions

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Viruses are small infectious agents that can replicate only inside the living cells of susceptible organisms. The understanding of the molecular events underlying the infectious process has been of central interest to improve strategies aimed at combating viral diseases of medical, veterinary and agricultural importance. Some of the viruses cause dreadful diseases, while others are also of interest as tools for gene transduction and expression and in non-poluting insect pest management strategies. The contributions in this book provide the reader with a perspective on the wide spectrum of virus-host systems. They are organized in sections based on the major topics covered: viral genomes organization, regulation of replication and gene expression, genome diversity and evolution, virus-host interactions, including clinically relevant features. The chapters also cover a wide range of technical approaches, including high throughput methods to assess genome variation or stability. This book should appeal to all those interested in fundamental and applied aspects of virology.

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