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REVIEW

The molecular and pathophysiological implications of hepatitis B X antigen in chronic hepatitis B virus infection

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SUMMARY

Hepatitis B virus is considered one of the most significant environmental carcinogens in humans. Because the mechanisms of HBV replication and the development of hepatocellular carcinoma (HCC) are partially known, HBV-associated pathogenesis remains a challenge to increase its understanding. Evidence suggests that the regulatory protein hepatitis B virus X (HBx) mediates the establishment and maintenance of the chronic carrier state. HBx is a multifunctional and potentially oncogenic protein that is conserved among mammalian hepadnaviruses; it is produced very early after infection and throughout the chronic phase. HBx exerts its effects by interacting with cellular proteins and activating various signaling pathways. HBx stimulates the transcription of genes that regulate cell growth, apoptosis, and DNA repair. It also interacts with proteasome subunits and affects mitochondrial stability. Moreover, HBx participates in processes that are associated with the progression of chronic liver disease, including angiogenesis and fibrosis. This review discusses the function of HBx in the life cycle of HBV and its contribution to the pathogenesis of HCC. Copyright © 2011 John Wiley & Sons, Ltd.

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Abbreviations used

AP-2, activating enhancer-binding protein 2-alpha; ATF2, activating transcription factor 2; ATF3, activating transcription factor 3; C/EBP, CCAAT/enhancer-binding protein; CA9, carbonic anhydrase 9; CBP, CREB-binding protein; cccDNA, covalently closed circular DNA; CD1, cluster of differentiation 1; Cdc2, cyclin-dependent kinase 1 or cell division control protein 2 homolog; Cdc6, cell division cycle 6 protein; CDK, cyclin-dependent kinase; Cdt1, chromatin licensing and DNA replication factor 1; CHB, chronic hepatitis B; CREB, cAMP response element-binding protein 1; CRM1, chromosome region maintenance 1; DDB1, damaged DNA binding protein 1; ERK, ELK-related tyrosine kinase; FAK, focal adhesion kinase; Fas, Fas antigen; CD-95; HBsAg, hepatitis B surface antibody; HBx, hepatitis B virus X protein; HBXIP, hepatitis B virus X protein interacting protein; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HepG2, human hepatocellular liver carcinoma cell line; HIF-1α, hypoxia-inducible factor-1 alpha; HSCs, hepatic stellate cells; HuH7, human hepatoma cell line; IκB, nuclear factor kappa-B inhibitor; iNOS, inducible NO synthase; JAK, Janus kinase; JNK, Jun N-terminal kinase; LHB, large hepatitis B surface protein; MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral signaling; MEK, mitogen-activated protein kinase; MHB, medium hepatitis B surface protein; MMP, matrix metalloprotease; MTA1, metastasis-associated protein 1; NFAT, nuclear factors of activated T cells; NFκB, nuclear factor kappa-B; NIH3T3, human embryo fibroblast cell line; pgRNA, pre-genomic RNA; PKB/Akt, protein kinase B; PTTG1, protooncogene pituitary tumor transforming gene 1; Pyk2, proline-rich tyrosine kinase; RBP5, retinol-binding protein 5; rcDNA, relaxed circular DNA; SAPK, stress-activated protein kinase; SHB, small hepatitis B surface protein; SMAD4, mothers against decapentaplegic, drosophila, homolog 4; Src, avian sarcoma viral oncogene; SREBP1, sterol regulatory element-binding protein-1; STAT, signal transducers and activators of a transcription; TFIIB, transcription factor IIB; TFIIH, transcription factor IIH; TGF-β, transforming growth factor beta; VEGF, vascular endothelial growth factor; WHV, woodchuck hepatitis virus; WHx, woodchuck hepatitis virus X protein.

HBV is a noncytopathic virus that was discovered in 1966 and belongs to the *Hepadnavirus* family of DNA viruses. HBV infection is a significant health problem—350 million people are chronically infected, despite vaccination and current antiviral strategies [1]. The HBV X protein, HBx, is a pleiotropic viral protein that is believed to have central functions in HBV biology and hepatic pathogenesis by modulating many viral and cellular functions. This review will focus on HBx activities, discussing key studies concerning its role on CHB disease progression and HCC development.

HBV GENOME

The preferential tropism of HBV is for hepatocytes, and its natural hosts are humans, although hepadnaviruses can infect other mammals and birds, such as herons, duck, goose, and cranes [2]. Hepadnaviruses have similar virion structures, consisting of a double-shelled particle that comprises a partially double-stranded rcDNA [1]. The long (minus) strand is approximately 3.2 kb, whereas the length of the complementary strand (positive) varies, ranging from 50% to 100% of the minus strand. The complementarity between the 5′ regions of both strands permits circularization of the genome. This region contains two direct repeated sequences—DR1 and DR2—that are necessary for template switches during viral replication and may be the precursor of DNA integration into the host cell genome [3].

HBV genomic organization is complex, because of its need to generate multiple proteins from a very small genome. It is a highly compact structure containing four overlapping ORFs—S, C, P, and X—all of which lie on the minus strand [4]. The generation of different viral proteins from the same reading frame is possible through alternative transcriptional start sites. Because the HBV genome is entirely coding, all regulatory signals, including the promoter, enhancer, transcriptional start sites, and polyadenylation signals, are embedded within coding regions. The preS/S ORF encodes the large, medium, and small surface proteins LHBs, MHBs, and SHBs, respectively, and the C/preC gene encodes the core and e antigens. Pencodes a multifunctional protein that harbors a viral polymerase that has polymerase RT and RNase activities and a terminal protein, which acts as a primer for HBV DNA synthesis. Finally, the X ORF encodes a 154-amino-acid polypeptide Mr 17,000 called HBx, which is required for *in vivo* infectivity [1,2,4] and other processes, which will be discussed in this review.

HBV TRANSCRIPTION, REPLICATION, AND VIRION ASSEMBLY

After viral attachment to the hepatocyte through the N-terminal region of LHBs and unknown cellular receptors, HBV fuses with the membrane and releases its nucleocapsid into the cytoplasm. The viral rcDNA is transported to the nucleus and transformed into a cccDNA [5] by cellular enzymatic machinery. This cccDNA is the template for the transcription of the pgRNA and other viral mRNAs by the host RNA polymerase II.

In hepadnaviruses, reverse transcription occurs through the retrotranscriptase-primed initiation of minus-strand DNA synthesis, which leads to the covalent linkage of RT to the packaging signal, an RNA stem-loop structure at the 5′ end of pgRNA called epsilon (ε). This event triggers encapsidation of viral RNA and polymerase into core particles. Following the assembly of subviral core particles, minus-strand DNA is reverse-transcribed into rcDNA during nucleocapsid maturation [4,6–9]. These mature capsids are assembled with the viral envelope proteins in the endoplasmic reticulum, effecting the formation of complete virions that can be released into the bloodstream (Figure 1). Alternatively, mature capsids with rcDNA can be recycled to the nucleus and amplify the nuclear cccDNA pool [2,4]. Tight control of envelope proteins is essential for effective virion production and the survival of infected cells, because an excess of LHBs over other envelope proteins leads to the accumulation of viral particles within the lumen of the ER and, consequently, cytopathic effects in hepatocytes [10].

HEPATITIS B VIRUS X PROTEIN

Hepatitis B virus X structure

The structural and biochemical properties of HBx are largely unknown because of difficulties in producing significant amounts of soluble protein. Despite repeated attempts to determine the structure of HBx by spectroscopy, no crystal model has been developed, likely reflecting that the protein is unstructured [11,12]. HBx is a protein of Mr 17,000 that shares homology with HBx in other mammalian hepadnaviruses but not with

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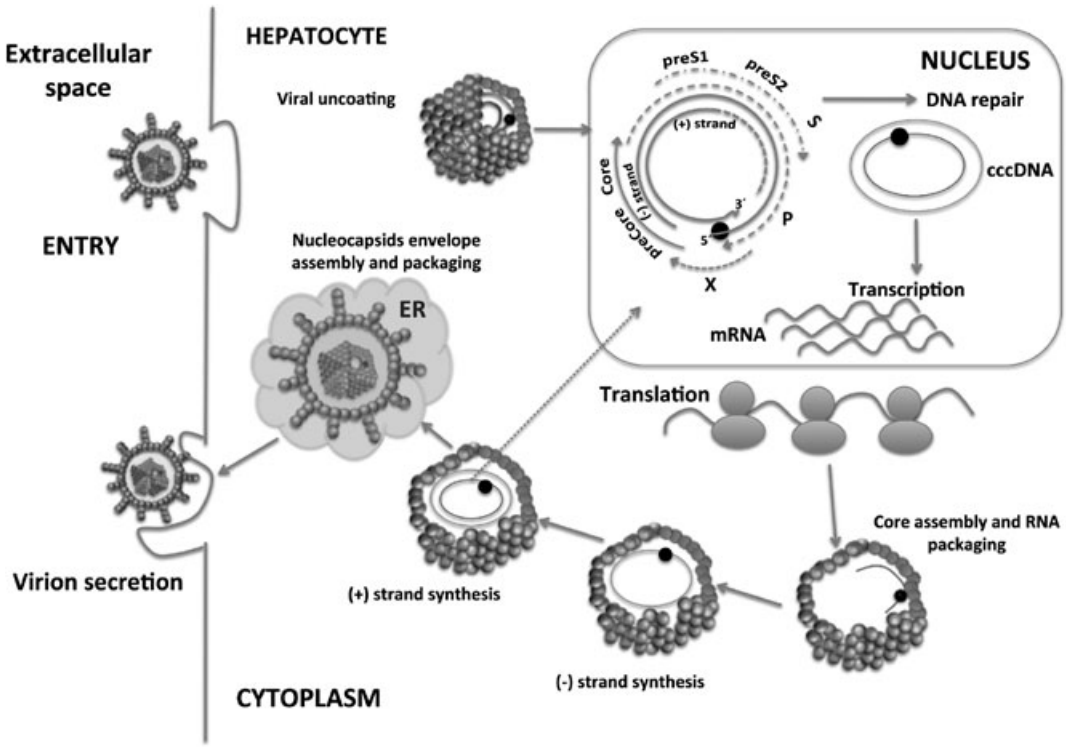

Figure 1. Schematic of HBV genome and life cycle. After viral attachment and entry into human hepatocytes, the nucleocapsid is uncoated and released into the cytosol. The viral genome is constituted by partially double-stranded circular DNA and contains four open reading frames encoding the viral polymerase (P), surface antigens (preS1, preS2, and S), precore, and HBx. In the nucleus, it becomes repaired and converted to cccDNA, which is used as a template for the transcription of all viral mRNAs. In the cytoplasm, the pregenome RNA, bound by polymerase, is encapsidated into core particles, followed by asymmetric synthesis of the DNA strands. The polymerase synthesizes a copy of the (–) strand DNA and degrades the RNA template. (+) strand DNA synthesis begins but is partially completed. After achievement of 50% or more of the (+) strand, nucleocapsids assemble with envelope proteins in the ER or are delivered to the nucleus for cccDNA amplification

any host protein [11,13]. A comparative analysis of x HBx from mammalian hepadnaviruses has revealed highly conserved areas, including domains that appear to adopt a helical structure in the amino-terminal and carboxy-terminal regions and a potential coil-to-coil motif. Two functional domains, at amino acid 69 and between amino acids 110–139, are critical for transactivation by HBx [14,15]. Notably, the first 50 amino acids of the amino terminus of HBx is a negative regulatory domain that allows the viral protein to regulate its transactivation [16]. HBx forms homodimers through disulfide bonds and post-translational modifications, such as acetylation, as described in human hepatoblastoma cells [17].

Localization

The detection and subcellular localization of HBx are debated. Most studies have observed that HBx exists primarily in the cytoplasm but that is detectable in the nucleus [18–22], whereas other reports have found HBx to be preferentially expressed in the nuclei of certain cell lines [23,24]. A model has been proposed to reconcile these findings, in which HBx is predominantly nuclear when expressed at very low levels and accumulates in the cytoplasm when its expression increases [19,25]. This dynamic distribution HBx might be vital, considering the various functions of HBx during the life cycle of HBV.

In this regard, cytoplasmic and nuclear HBx has recently been shown to mediate viral replication [19,25]. Other studies have demonstrated the capacity of nuclear HBx to restore the replication of HBx-deficient HBV [26]. This dual localization might influence transcriptional activation in the nucleus and viral replication in the cytoplasm. Although the molecular mechanisms of HBx localization are not understood, recent studies have shown that HBx is exported from the nucleus by interacting

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with the nuclear export receptor, CRM1. HBx overexpression inactivates CRM1, promoting nuclear accumulation and activation of transcription factors, such as NFκB [27]. Notably, IκB-α has been described to regulate the nuclear translocation of HBx [28].

Transactivation activity of HBx and regulation of signal transduction

The most extensively studied property of HBx and that has been widely recognized in an undeniable manner is its capacity to transactivate. This activity is believed to be crucial for the development of liver cancer, because it is involved in HBV transcription and replication [29], upregulating many genes that mediate oncogenesis, proliferation, and immune responses [30]. HBx is a weak transactivator that stimulates transcription from many viral and cellular promoters (Figure 2), despite its inability to bind to DNA directly [31,32].

HBx associates with several components of basal transcriptional machinery, including TFIIB, TFIIH, RBP5, and modulates RNA polymerase activity [33,34]. It interacts with transcription factors, such as CREB, ATF-2, ATF-3, NFAT, C/EBPβ, SMAD4, SREBP1, and AP-2, to modify their activities [35–40]. For instance, HBx interacts *in vitro* and *in vivo* with CREB, a member of the basic leucine zipper (b-Zip) family of transcription factors. This interaction increases the affinity of CREB for its binding site in DNA, enhances *in vitro* occupation of HBV enhancer I by ATF/CREB, and correlates with CREB-dependent transcriptional activation by HBx *in vivo* [39]. Recently, HBx has been demonstrated to interact and cooperate with CBP/p300 to synergistically enhance CREB activity [41]. Thus, the ability of HBx to interact with these proteins might constitute a mechanism by which it regulates transcription. The effect of HBx on CREB/ATF might be relevant for liver pathology, because the CREB/ATF family regulates liver metabolism, proliferation, and tumorigenesis [42]. Similarly, studies in transgenic models have shown that CREB regulation by HBx could also govern HBV transcription, because HBV enhancer I and PreS2 contain CREB

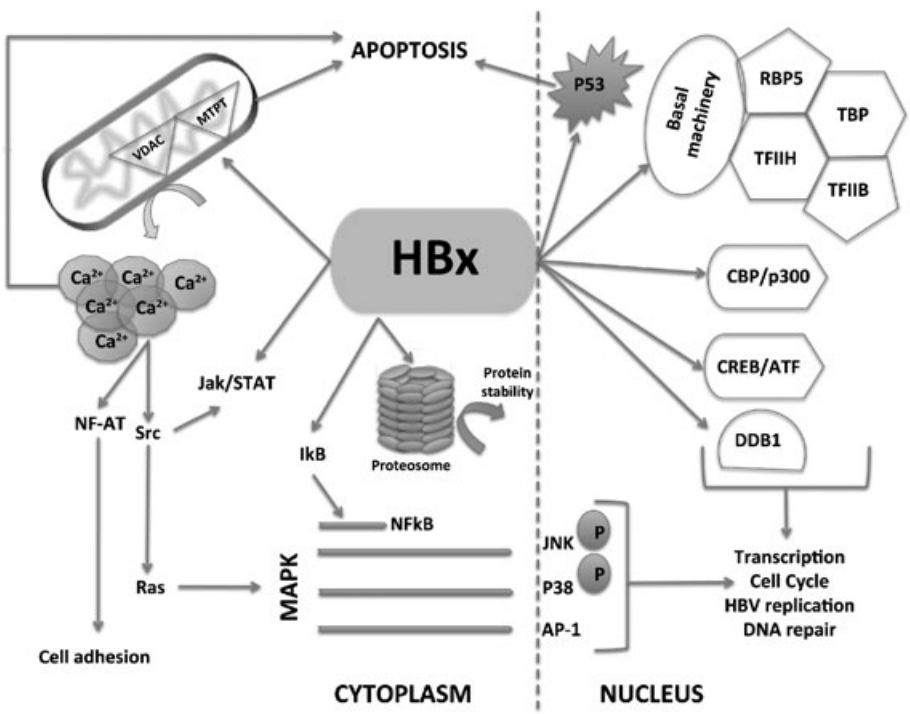

Figure 2. Multiple functions of HBx. HBx activates viral and cellular transcription through direct binding to transcription factors, co-activators, and components of the basal transcription machinery. HBx can also trigger transcription indirectly through the release of calcium to the cytosol, which stimulates signaling pathways, such as NFκB, JAK/STAT, and MAPK. In addition, HBx interacts with proteasome subunits, regulating protein degradation and stability. Moreover, HBx may interact with various cellular components, such as mitochondria, DDB1, and p53, regulating apoptosis, cell cycle, transformation, and viral replication

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binding site-like sequences [43]. As discussed, HBx activates NFκB, although the mechanism by which this occurs remains unknown. It is unclear whether NFκB activation by HBx requires protein C or Ras [18,44–46] and whether HBx acts directly on NFκB, modulates phosphorylation and degradation of the NFκB inhibitor IκB, or is activated by upstream signaling pathways [28,46]. These possibilities might be attributed to varying degrees of NFκB activation in different cells and experimental models.

HBx controls several cytoplasmic signal transduction cascades, including PKB/Akt, ERK, SAPK/JNK, and p38 [18,47,48]. This transactivation requires cytoplasmic HBx, as shown in many cell lines, including those of liver origin [18,48,49]. Initiation of the Ras-Raf-MAPK, JNK, p38, and JAK/STAT pathways by HBx requires the activation of nonreceptor tyrosine kinases of the Src family [50]. Dr. Lopez Cabrera's group has proposed several mechanisms by which Src activation may be regulated by HBx [37]. HBx modulates cytosolic calcium levels to stimulate upstream activators of Src kinases—FAK and Pyk2 [51,52]. The regulation of intracellular calcium levels by HBx is believed to be critical not only for the activation of MAPK pathways but also for viral replication through the activation of Src [48,51–53]. HBx could regulate cytosolic calcium levels through its association with mitochondria [54,55], which disrupts and depolarizes mitochondrial architecture [55,56]. Activation of Src by HBx also destabilizes adherent junctions in liver cell lines [57].

HBx and HBV life cycle—essential or accessory?

The high conservation in *Hepadnavirus* genomes between humans and other mammals suggests that HBx regulates the viral life cycle [58,59]. In certain experimental models, such as the HuH7 cell line, HBx-deficient HBV genomes are able to replicate, but viral replication is inhibited in HepG2 cells [29,59,60]. In contrast, *in vivo* experiments using the highly related WHV have demonstrated that WHx is necessary for WHV infection in woodchucks. These studies show that WHV genomes fail to replicate and that neither infection nor viremia is detectable when WHx expression is abolished [61]. Yet, another group found that WHV mutants for WHx replicated at very low levels [62].

HBx ability to enhance viral replication *in vivo* was further supported by a report in which HBV transgenic mice that lacked HBx were crossed with HBx-expressing transgenic mice [63]. Similarly, Keasler and colleagues using a plasmid-based HBV replication assay provided evidence on the importance of HBx in HBV replication *in vitro* and *in vivo* [26]. In an acute hepatitis model, mice infected with a plasmid containing a HBV genome deficient for HBx showed compromised viral replication that was restored to wild-type levels by the transfection of HBx targeted to the nucleus [26]. The same group demonstrated that nuclear localization of HBx, using the same plasmid-based assays, is critical for attaining maximal levels of capsid-associated viral DNA in transfected HepG2 cells [26]. The importance of HBx in the context of HBV infection was recently demonstrated by Tsuge and colleagues where only human hepatocyte chimeric mice develop a measurable viremia in HBx-expressing livers [64].

Epigenetic events modulated by HBx influence cccDNA function and HBV replication by the inhibition of cccDNA deacetylation [60]. A recent work has also demonstrated that HBx is needed for productive HBV infection favoring the acetylation of histones associated to cccDNA and, therefore, the synthesis of viral RNAs in the context of the viral replication [65].

HBx and the cell cycle

Activation of signal transduction pathways such as MAPK, JNK, and Src, by HBx can accelerate cell cycle by inducing the exit of quiescent cells from G0 and the transition from G1 to S phase and G2 to M phase [66]. The ability of HBx to induce cell cycle progression is influenced by additional factors. HBx-transfected cells that are supplemented with serum accelerated transit through cell cycle checkpoints compared with cells that lack serum, which exit from G0 but stall at the G1/S checkpoint [67]. These results suggest that additional signals, such as those that are elicited by growth factor receptors, are needed for HBx to enhance cell proliferation.

HBx modulates the levels of the cell cycle regulators p16, p21, p27, cyclin D1, cyclin A, and cyclin B1 and increases the activity of CDK2 in various cell lines and primary hepatocytes, although the precise effects of HBx vary according to experimental conditions [47,68–73]. The comparison of

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different HBx-expressing hepatocyte lines from the same parental cell line reflected that differentiated hepatocytes experienced HBx-dependent G1, S, and G2/M phase progression and induction of cyclins D1, A, and B1 and Cdc2, whereas dedifferentiated cells showed HBx-dependent entry into G1 and S phase by pausing early in S phase. Further, dedifferentiated cells exhibited HBx-induced expression of the CDK inhibitor p21 [48].

These *in vitro* studies suggest that HBx renders hepatocytes sensitive to other potentially carcinogenic signals, which, when combined with immune viral responses and its interaction with other proteins, can affect hepatocyte transformation (Figure 3).

The effects of HBx on hepatocyte regeneration and cell cycle progression have been explored *in vivo* using HBx-transgenic mice, although the results have not been consistent. Two studies demonstrated that HBx impairs hepatocyte regeneration after partial hepatectomy and causes a delay in the G1/S transition [74,75]. In contrast, another study reported enhanced hepatocyte proliferation and apoptosis in HBx-transgenic mice [67]. Hodgson *et al*. recently reported that hepatocyte division was unaffected by HBx in other HBx-transgenic mouse model, although it induced a fraction of hepatocytes to enter the cell cycle prematurely [75]. To determine the exact impact that HBx has on cell cycle progression, Gearhart and colleagues examined the effect of HBx on cell proliferation pathways in HBV replication in cultured primary rat hepatocytes. Their results suggest that HBx induces quiescent hepatocytes to enter G_1 phase and that calcium is necessary for HBx-induced HBV replication and modulation of hepatocyte proliferation [74].

These studies indicate that HBx alters cell proliferation, particularly by inducing re-entry of normally quiescent hepatocytes into the cell cycle. Thus, an understanding of the molecular mechanisms of HBx-induced cell cycle progression and its effects on normal hepatocyte physiology will help identify the mechanisms that link chronic HBV infection to the development of liver cancer.

Despite these controversies, a consensus exists on a specific effect of HBx on the cell cycle: HBx expression tends to increase the formation of multinucleated cells and dysregulate the progression of mitosis, favoring chromosomal rearrangement and micronuclei formation, thus leading to the

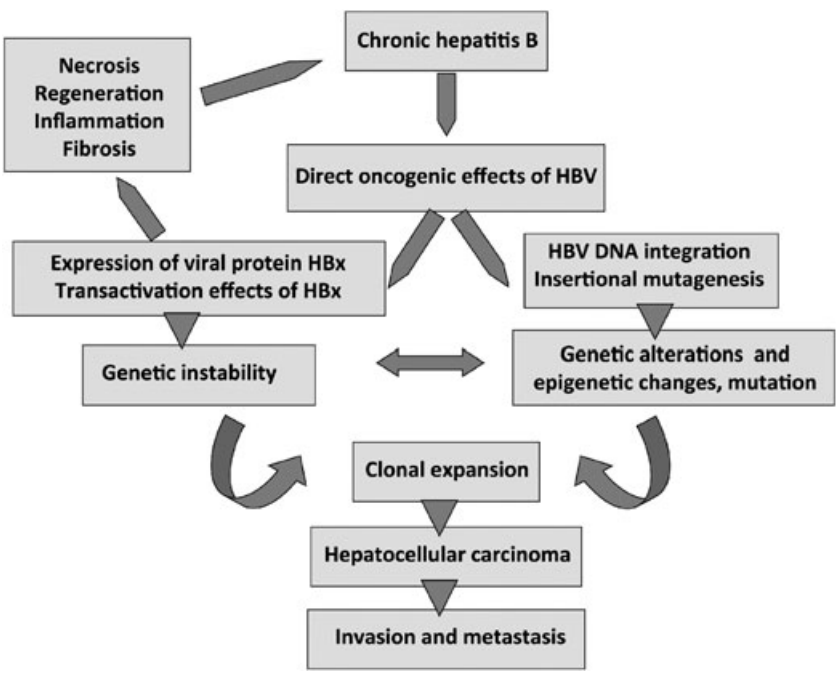

Figure 3. Mechanisms of HBV-associated chronic hepatitis and hepatocarcinogenesis. Prolonged viral protein expression and integration of the viral genome into host DNA can directly induce genetic instability. These processes could be coupled to loops between hepatocyte regeneration and necrosis, promoted by the host's immune response, also contributing to the development of genetic alterations. Ultimately, these two mechanisms could affect the development of hepatocarcinoma

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loss of genomic integrity [76–80]. The mechanisms that underlie these effects are diverse. Some involve activation of the Ras-MEK-MAPK signaling pathway by HBx [81]. Other groups have shown that HBx induces genetic instability by interacting with DDB1 or with proteins that regulate centrosomal integrity, such as CRM1 and HBXIP [76,79,82]. HBx can bind BubR1, a component of the mitotic checkpoint complex, promoting its dysregulation [80].

Through PTTG1 accumulation, HBx can also affect the progression of mitosis and chromosomal segregation, because PTTG1 overexpression is associated with mitotic disruption, promoting abnormal cytokinesis without chromosomal separation [83,84]. HBx expression also induces DNA re-replication, DNA damage, and polyploidy, increasing the activity of Cdc6 and Cdt1 replication factors and reducing the expression of geminin, a negative regulator of DNA replication [80]. The concurrence of cell cycle progression with chromosomal rearrangements that are induced by the viral transactivator might explain the pro-oncogenic potential of HBx.

HBx and apoptosis

The relationship between HBx and apoptosis is a topic of HBV biology that illustrates its complex and paradoxical effects.

HBx prevents apoptosis by interfering with pathways that are activated by Fas and TGF-β [85,86] or by interacting directly with p53 [87,88] or caspase-3 [89,90]. Various studies have proposed that activation of the PI3K cascade mediates the suppression of apoptosis by HBx. Apoptosis is inhibited in serum-deprived or pro-apoptotic drug-treated Chang cells transiently transfected with HBx [89,90]. Similarly, in stably transfected Hep3B cells, HBx inhibits TGF-β-induced apoptosis [86]. HBx expression prevents Fas-mediated apoptosis in DP-16 cells (a mouse erythroleukemia cell line), mouse embryonic fibroblasts, HepG2 hepatoblastoma cells, and Chang Liver cells that constitutively express HBx [85]. Also, HBx reduces apoptosis in REV2 rat fibroblast cells in response to serum deprivation, FasL, or TNF-α [89]. In another anti-apoptotic mechanism, HBx decreases caspase activity through its association with survivin, an anti-apoptotic protein that is overexpressed in most human cancers [90].

NFκB pathway has been examined as a potential mechanism of the anti-apoptotic effects of HBx. In hepatocytes, NFκB activation by HBx is linked to the inhibition of TNF-α-stimulated and Fas-stimulated apoptosis [28,44,46,55]. However, these effects on cell viability could depend highly on the cellular context.

In contrast to the proposed anti-apoptotic functions of HBx, several reports have observed increased sensitivity to pro-apoptotic stimuli following mitochondrial damage by HBx [91,92]. Cytochrome C release and mitochondrial aggregation have been proposed as mechanisms of these effects [56,92,93]. As discussed, HBx affects mitochondrial function by interacting with the voltage-dependent anion channel, a component of the mitochondrial permeability transition pore, altering cytosolic calcium levels [55] and depolarization of the mitochondrial membrane [53].

Cellular homeostasis and genomic stability is affected by ROS (Reactive Oxygen Species) because they react with biomolecules such as DNA, proteins, and lipids, generating oxidative damage. However, ROS are also critical for cellular functions; thus, optimal ROS levels must be properly regulated in order to avoid oxidative DNA lesions that can lead to DNA mutations and chromosome abnormalities as are frequently observed in the liver of HBV-infected patients. In this regard, previous studies have revealed the interaction of HBx protein with the membrane proteins of mitochondria, altering the mitochondrial membrane potential in hepatoma cell lines. In addition, level of mitochondrial ROS and lipid peroxide production was also shown to be increased by HBx, which leads to, by unknown mechanisms, the constitutive activation of STAT-3 and NFκB [138]. Recently, it has been revealed in HBx chimeric mice and HepG2 cells that HBx-induced ROS stimulate Akt pathways via oxidation and inactivation of PTEN (Phosphatase and Tensin Homolog). The authors conclude that the positive regulatory loop established between HBx and ROS may promote tumorigenesis [94].

However, no mitochondrial aggregation is observed during HBV replication or viral infection, suggesting that this effect is due to overexpression of HBx. In HBV replication, HBx renders cells receptive to various pro-apoptotic stimuli. HBx increases the sensitivity to apoptosis, primarily to TNF-α, by activating the JNK and Myc pathways [56,95]. Additionally, HBx appears to induce apoptosis in p53-dependent [96,97] and p53-independent manners [98].

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There are also contrasting effects of HBx expression on apoptotic pathways in HBx-transgenic mouse models. In mice in which HBx is expressed under the control of the human α-1-antitrypsin regulatory region, no increase in hepatocyte apoptosis is observed; further, on crossing these mice with Bcl-2-overexpressing transgenic mice, HBx inhibited Bcl-2-mediated protection from apoptosis [99]. Alternatively, apoptosis might be a consequence of other actions of HBx that are deleterious to the cell, such as deregulation of the cell cycle. Regardless, these hypotheses have not been examined in an experimental infection model, and the induction of apoptosis by HBV infection has not been documented *in vivo*.

ETIOPATHOGENIC ROLE OF HBX

Although HBx transactivation capacity has been widely recognized as well as its important role for HBV replication, its direct implication on liver pathophysiology is still debatable and highly dependent on experimental system, which in any case is the natural host for HBV infection.

HBx and the immune response

HBV is a noncytopathic virus; thus, HBV-associated liver damage is a consequence of a chronic cytolytic immune response against infected hepatocytes [100].

HBx induces the expression of genes that are associated with the immune response. Paradoxically, HBx upregulates proinflammatory factors, such as TNF-α, iNOS, and IL-6 [101,102], but not immunosuppressive proteins, such as IL-10. TNF-α is a pivotal cytokine in host defense and exerts broad effects on the innate and adaptive immune system. This pleiotropic cytokine mediates several pathophysiological processes in the liver, including regeneration, chronic hepatitis, cirrhosis, and the development of hepatocarcinoma [103].

In hepatocytes, TNF-α triggers proliferative and apoptotic signaling pathways; ultimately, the response of a cell to TNF-α depends on a fine balance between both pathways. Similarly, HBx induces the expression iNOS, which also has a dual effect on hepatocytes [102]—it inhibits viral replication yet promotes IL-6-mediated hepatocyte proliferation [104]. Thus, HBx can modulate the induction of several cytokines, altering the balance between proliferation and apoptosis. Recently, HBx was shown to attenuate the antiviral response of the innate immune system by promoting the degradation of MAVS protein, an essential component of the virus-activated signaling pathway that activates NFκB and IFN regulatory factor-3 to induce the production of type I IFN [105]. Consequently, HBx-induced degradation of MAVS impairs the induction of IFN-β and the innate antiviral response [105], preventing the death of infected hepatocytes and allowing HBV to survive and evade immune surveillance.

HBx role in hepatic fibrosis and angiogenesis

Conclusions concerning HBx role on angiogenesis and fibrosis are limited because they are mainly supported on results achieved from *in vitro* experiments, reflecting the need of further *in vivo* corroboration. There is no evidence that HBV infects HSCs, whose activation is central in the development of fibrosis and cirrhosis in chronic liver diseases [106]. The few studies that have examined whether HBx is linked to hepatic fibrosis have assumed that HBV infects hepatocytes that release profibrogenic substances (cytokines and reactive oxygen species) to activate neighboring HSCs [107].

In this regard, our group has reported that HBx induces paracrine activation and proliferation of HSCs through TGF-β [108], a major profibrogenic cytokine [106,109]. Further, HBx stimulates and enhances collagen-1 secretion and MMP-2 by HSCs, promoting the remodeling of extracellular matrix [107]. These results are consistent with those showing that HBx regulates MMP-1, which in turn activates MMP-2 and upregulates MMP-9, suggesting that HBx alters the expression of several MMPs during the development of fibrosis, cirrhosis, and cancer [110,111].

HBx has been implicated in angiogenesis and metastasis as HCC cells become aggressive, acting as a novel modulator of hypoxia-induced angiogenesis in CHB. HBx enhances the transcription of VEGF, a potent angiogenic cytokine, by upregulating the transcription of HIF-1α [112,113]. Increased expression of CA9, one of the most hypoxia-responsive genes, shows a new additive effect of HBx over HIF transactivation [114].

Further, HBx increases HIF-1α stability by inducing MTA1 and HDAC genes. The MTA1/HDAC1 complex increases HIF-1α deacetylation and, consequently, HIF-1α stabilization, constituting a possible mechanism of HBV-associated angiogenesis [115]. As described, other pro-angiogenic molecules that

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are regulated by HIF-1α, such as MMP-2, are induced by HBx [116,117].

By enhancing expression of MMP-1, MMP-3 and MMP-9 HBx could favor metastasis in addition to angiogenesis, and thus increase the invasive potential of HCC cells [57,110,111,118,119]. Moreover, our group has observed increased expression of angiopoietin-2, a critical regulator of vascular development and angiogenesis, in liver tissue of chronically infected patients, HBx-expressing hepatic cell lines and HSC stimulated with conditioned medium from HBx-expressing hepatocytes [120]. These data suggest a positive crosstalk between HBx and pro-angiogenic mechanisms, implicating HBx in CHB progression to HCC, a highly vascularized malignant tumor (Figure 4).

Uncertain influence of HBx on hepatocarcinoma development

HBx association with the development of HCC was first proposed from indirect evidence that HBsAg carriers, who present with active viral replication, chronic hepatitis, and HCC, frequently express antibodies against HBx [57,105,118,121,122]. HBx sequences integrate into host DNA in HBV-associated HCC, occurring in 85% to 90% of cases.

Several groups have reported that the 3′-end of the X gene is frequently deleted in the HCC cells after HBV integration into the host genome, leading to C-terminally truncated HBx [7,8,123,124]. Although it is unknown whether these mutants regulate the development of HCC, a recent study has reported that they lose their pro-apoptotic activity. Moreover, truncated HBx, but not full-length HBx, is able to transform liver cell lines and might promote liver tumors by enhancing cell proliferation [122].

There is little evidence whether HBx transforms directly in animal and cell culture models; existing results are conflicting because transformation of cells expressing an intact hepadnavirus genome has generally not been observed. Despite this, there

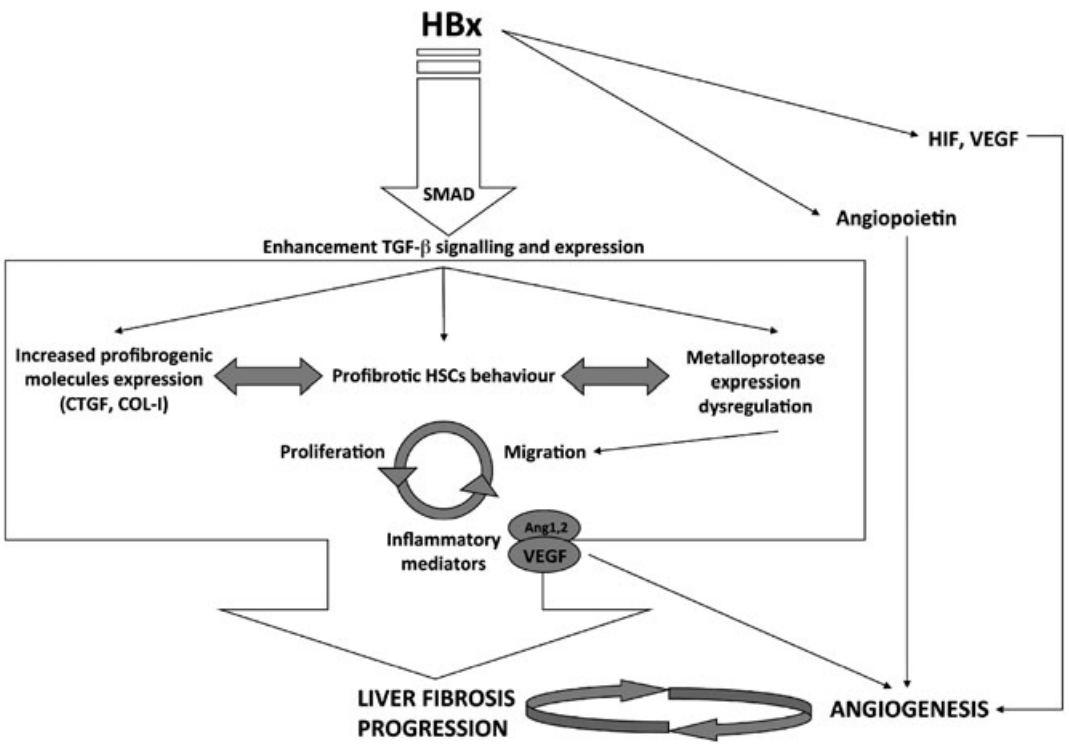

Figure 4. Schematic of HBx and its relationship with fibrosis and angiogenesis. HBx induces TGF-β, the most important mediator of fibrosis, which leads to the initiation of profibrotic events, such as hepatic HSC activation, collagen-1 accumulation, and impaired extracellular matrix remodeling, due to dysregulation of metalloprotease balance and increased expression of profibrotic molecules. HBx might govern angiogenesis inducing angiopoietins and VEGF expression by stabilizing HIF. Furthermore, activated HSCs secrete inflammatory mediators that can accelerate angiogenesis. Because many of these events are linked, these established feedback loops make fibrosis and angiogenesis difficult to separate. Thus, angiogenesis and fibrosis are parallel events that propel each other, favoring liver injury

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are studies that show HBx transforming activity over cell lines immortalized by simian virus 40 large T antigen [125,126], over primary human fibroblasts (NIH3T3) in association with Ras activity [127,128], and over immortalized rodent hepatocytes [129]. In contrast, other studies show that HBx apoptotic effects suppress transformation mediated by oncogenes in primary rat embryo fibroblasts and in NIH3T3 [130,131]. However, disadvantage of these observations arise from the fact that they were made primarily on overexpression of HBx, whereas its expression in chronically infected livers is low.

In the same way, analysis of HBx oncogenicity in transgenic mice show divergent results that might be attributed to disparities in mouse strain, transgene sequences, and integration sites, as well as HBx lifelong expression and levels [132]. Some experimental evidence points to the viral protein HBx as a causative agent for the cellular transformation, because some strains of HBx-transgenic mice have a strong tendency to develop liver cancer [133,134]. For example, HBx overexpression in cell embryos from CD1 mice increased frequency of HCC development [134,135]; in contrast, transgenic mice expressing lower levels of HBx did not show such frequency of liver tumor compared with normal CD1 mice [135].

In addition, other studies in HBx-transgenic mice have failed to confirm the elevated risk for liver cancer [136,137]. Thus, such results suggest that HBx is not oncogenic *per se*; the necro-inflammatory and renewal loop in the liver is a highly mutagenic process by itself. Therefore, it would be reasonable to consider HBx as a tumor promoter. In this regard, several groups have described that HBx expression in murine liver induces proliferation and apoptosis, cooperating with c-myc or chemical carcinogens to promote hepatocarcinogenesis [138–140]. Additionally, Wang and colleagues have demonstrated that HBx promotes HCC in p21-deficient mice [141]. Finally, a recent work found that HBx favor HBV-induced hyperplasia through the cooperation with tumor suppressor genes and oncogenes, but interestingly, HBx did not cooperate with constitutively active NRAS in driving liver tumorigenesis [142].

CONCLUSIONS

HBx has significant functions in all stages of HBV infection and during the progression of hepatitis to fibrosis and HCC through the transactivation of a numerous cellular signaling pathways. Despite the use of different experimental approaches, transgenic animals, and immortalized cell lines to study HBx function, the results have been quite contradictory. Greater effort is needed to better define the molecular mechanisms through which HBx contributes to CHB-associated pathophysiologic processes, such as angiogenesis, fibrosis, and development of HCC. New tools, especially reliable animal models, are needed to determine how such a small protein can possess a wide variety of actions affecting crucial viral aspects as well as cellular and tissue homeostasis. Additionally, a greater understanding of these complex HBx–host interactions may foster the development of novel antiviral therapies.

CONFLICT OF INTEREST

The authors have no competing interest.

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