

R E V I E W



Wild type HBx and truncated HBx: Pleiotropic regulators driving sequential genetic and epigenetic steps of hepatocarcinogenesis and progression of HBV-associated neoplasms

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SUMMARY

Hepatitis B virus (HBV) is one of the causative agents of hepatocellular carcinoma. The molecular mechanisms of tumorigenesis are complex. One of the host factors involved is apparently the long-lasting inflammatory reaction which accompanies chronic HBV infection. Although HBV lacks a typical viral oncogene, the HBx gene encoding a pleiotropic regulatory protein emerged as a major player in liver carcinogenesis. Here we review the tumorigenic functions of HBx with an emphasis on wild type and truncated HBx variants, and their role in the transcriptional dysregulation and epigenetic reprogramming of the host cell genome. We suggest that HBx acquired by the HBV genome during evolution acts like a cellular proto-onc gene that is activated by deletion during hepatocarcinogenesis. The resulting viral oncogene (v-onc gene) codes for a truncated HBx protein that facilitates tumor progression. Copyright © 2015 John Wiley & Sons, Ltd.

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INTRODUCTION

Alongside alcohol consumption, exposure to aflatoxin B1, hemochromatosis and hepatitis C virus (HCV) infection, human hepatitis B virus (HBV) infection is the main cause of hepatocellular carcinoma (HCC) worldwide. Chronic HBV infection accounts for more than half of HCC cases, and more than 80% of HCCs in highly endemic countries like China. HBV, a small DNA virus from the

Orthohepadnavirus genus of the *Hepadnaviridae* family, has a partially double-stranded, relaxed-circular genome (RC-DNA). After infecting non-dividing hepatocytes, HBV RC-DNA is converted into covalently closed circular DNA (cccDNA) with the contribution of the cellular DNA repair machinery, followed by transcription and replication of the genome. HBV replication does not necessarily involve integration of HBV DNA into the cellular genome [1,2] (reviewed by [3,4]).

HBV causes acute, mostly resolving or, at a smaller percentage, persistent liver disease. Approximately 2 billion people are infected, whereas 350 million are estimated to be chronic carriers of HBV which means that their infected hepatocytes express hepatitis B surface antigen (HBsAg) and shed HBsAg and viral DNA into their blood stream. Chronic HBV infection frequently results

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Abbreviations used

cccDNA, covalently closed circular DNA; DL-DNA, duplex linear DNA; HBc, HBV core gene or protein; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RC-DNA, relaxed circular DNA; trHBx, truncated HBx; TF, transcription factor; wtHBx, wild type HBx.

in liver cirrhosis which facilitates the development of HCC in a multistep process. Compared to non-infected individuals, the risk of HCC development in chronic HBV carriers and patients with chronic liver disease is about 30 times and 100 times higher, respectively. Mass vaccinations in high incidence areas successfully curbed HCC incidence [5]. Therefore, HBV is clearly causative for hepatocarcinogenesis. However, HBV does not code for a generally acknowledged oncoprotein, although the product of the viral HBx gene may play such a role. Thus, the molecular mechanisms of HBV-associated hepatocarcinogenesis are still a major riddle of tumor virology (reviewed by [6,7]).

Gene expression patterns, genetic and epigenetic modifications distinguish HBV-associated HCC from HCC of other origins. Integration of the HBV genome into the host cell DNA, modulation of apoptotic and cell signaling pathways by the pleiotropic, full length HBx protein (wild type HBx, wtHBx) and its C-terminal truncated variants (trHBx proteins) or other viral proteins, epigenetic dysregulation, transcriptional reprogramming, chronic inflammation, generation of reactive oxygen species and immune reactions all contribute to HBV-associated carcinogenesis (reviewed by [6–10]). Here we wish to focus on the contribution of wtHBx comprising 154 amino acid residues and its C-terminal truncated variants (trHBx) to HCC development. trHBx proteins were regularly observed in HCCs and differ significantly from wtHBx in their biological activities (reviewed by [10]). A similar phenomenon was observed during the generation of certain oncoproteins encoded by retroviral oncogenes, too. It is well documented, that the 5' or 3' sequences of cellular proto-onc genes transduced by retroviruses as v-onc genes are frequently deleted [11]. Such deletions may profoundly affect the properties of the encoded proteins, as exemplified by the C-terminal truncated variant of the cellular c-myc (myeloblastosis) protein in mice [12]. Similarly, a truncated Cbl (Casitas B-lineage lymphoma) protein also acts as the viral oncoprotein of a murine retrovirus [13]. The C-terminal truncation that generated the v-cbl oncogene altered the intracellular localization, DNA binding properties and transforming potential of the Cbl protein [14]. Thus, one possibility certainly is that wtHBx may be activated by 3'-deletion to a viral oncogene coding for trHBx which contributes to oncogenesis.

HBV GENOME: TRANSCRIPTIONAL REGULATION

Both covalently closed, circular HBV genomes persisting as episomes in the host cell nucleus and HBV genomes integrated into the host cell DNA are transcribed by the cellular RNA polymerase II (Pol II). From the episomal genomes, Pol II generates both viral mRNAs translated in the cytoplasm and viral pregenomic RNA molecules (pgRNAs) that act as the template for the synthesis of minus-strand viral DNA by the viral reverse transcriptase, followed by plus strand DNA synthesis inside the capsid [15]. In addition to cellular transcription factors (TFs) and the epigenetic regulatory machinery of the host cell, the viral wtHBx protein is also involved in the regulation of HBV gene expression.

Host factors regulating HBV transcription: the metabolovirus model

A multitude of cellular TFs are involved in the regulation of HBV gene transcription. Most of them are involved in the regulation of major hepatocellular metabolic pathways, e. g. gluconeogenesis, bile-acid production and fat accumulation. This has led Shaul and colleagues to propose the term “metabolovirus” for HBV and suggest that viral gene expression may be regulated by nutritional cues [16,17]. The response to nutritional signals may be mediated by peroxisome proliferator-activated receptor-gamma coactivator 1alpha (PGC-1 α) which is a central coactivator of glucose metabolism [18]. PGC-1 α synergizes with the forkhead factor FoxO1 and the nuclear receptor HNF-4 α in the induction of key metabolic genes in hepatocytes. Similarly, both, FoxO1 and HNF-4 α bind to both viral enhancers and the core promoter and coactivate viral gene transcription together with PGC-1 α [19]. The strong dependency of viral gene transcription on liver specific TFs may constitute the most important post-receptor restriction element for the liver tropism of HBV. Besides PGC-1 α , the viral wtHBx protein is the other important coactivator of HBV gene transcription. By binding together with CREB and C/EBP to HBV enhancers, wtHBx upregulates HBV transcription [20–22].

Epigenetic regulation of the HBV genome

HBV gene expression is typically controlled by two enhancers, four promoters and three CpG islands

(Figure 1). CpG island 1 (CGI 1) is associated with the HBs gene start codon, CGI 2 is associated with both enhancers and the core (HBc)- and X gene (HBx)-promoters, and CGI 3 is associated with the polymerase gene start codon [22–24]. However, the numbers and distribution of CGIs in HBV genomes differ to some extent depending on the viral genotype [25].

Clearly, HBV gene expression is regulated by epigenetic mechanisms, including DNA methylation and histone modifications. The encapsidated viral DNA was unmethylated, just like viral DNA in the liver of patients with chronic active hepatitis, whereas in occult HBV infection as well as in HCC and hepatoma cell lines, a variable methylation pattern of HBV DNA was observed [24,26–32]. Methylation of the HBs and HBc genes correlated with a lack of expression, whereas the HBx gene was frequently unmethylated even in genomes which were highly methylated at other loci [31]. Methylation of transfected viral genomes strongly decreased HBs and HBc gene transcription from viral cccDNA in

cultured hepatoma cells and silenced integrated viral genomes in HBV-transgenic mice [33].

The viral genome is not only subject to CpG-methylation, but it also actively induces DNA methyltransferases (DNMTs). Hepatoma cell lines responded to HBV infection with an upregulation of DNMTs resulting in methylation-mediated transcriptional silencing of the viral genome and suppression of virus replication [34]. Methylation of HBV DNA is regarded as an antiviral cellular defense mechanism. However, silencing of cellular tumor suppressor genes by promoter hypermethylation may be an inadvertent side effect of this defense mechanism which then may contribute to tumorigenesis [34] as discussed later (see “Epigenetic dysregulation in HBV infected cells: The role of wtHBx”).

The nuclear HBV genome is organized into nucleosomes like a minichromosome [35]. In addition to histones, the viral protein HBc is also part of the viral chromatin [36]. HBc, a positive regulator of HBV transcription, preferentially bound to CGI 2, and facilitated TF binding and hypomethylation in liver biopsy samples [37]. Transcriptionally active cccDNA genomes were associated with acetylated histones H3 and H4 and histone acetyltransferases (HATs) including p300, CBP and PCAF/GCN5. Histone deacetylases (HDAC) HDAC1 and hSirt1 were also recruited to cccDNA. Indirect binding of HBx to the viral cccDNA enhanced viral replication and transcription in hepatoma cells and human biopsy samples [38–40]. In primary hepatocytes or differentiated hepaRG cells, HBx relieved histone methyltransferase SETDB1-mediated transcriptional repression of viral cccDNA [41]. IFN α -treatment of HBV-carrying cells or hepatocytes of humanized transgenic mice silenced HBV transcription and replication directly via an interferon-stimulated response element (ISRE) located at the HBx promoter/enhancer 1 region of the viral genome. IFN-treatment resulted in histone deacetylation at the cccDNA in hepatoma cells and in human hepatocytes colonizing transgenic mice. In addition to HDAC, the polycomb group proteins EZH2 and YY1 were also recruited to the HBx promoter [42].

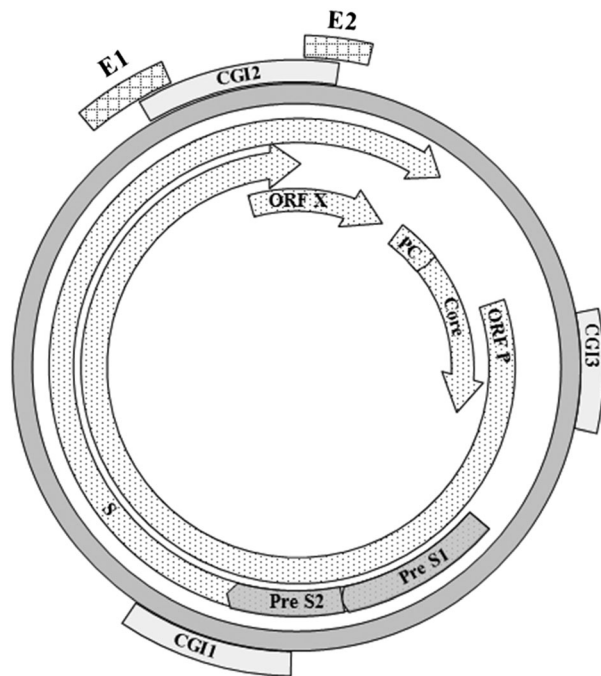


Figure 1. The HBV genome: enhancers, CpG islands and open reading frames. The circular HBV genome is represented by a dark grey line. E1, E2: position of enhancers. CGI1, CGI2 and CGI3: position of CpG islands. Arrows indicate open reading frames. ORF X: HBx; PC: precore protein; core: core protein; ORF P: polymerase protein; Pre S1: domain of the large surface protein; Pre S2: domain of the large and middle surface proteins; S: major surface protein

INTEGRATION OF HBV DNA INTO THE CELLULAR GENOME

Reverse transcription of pregenomic viral RNA in cytoplasmic nucleocapsids of hepadnavirus infected cells is initiated by the HBV reverse transcriptase that

also acts as a priming protein. The synthesis yields predominantly relaxed-circular HBV DNAs [1,43]. There is another pathway for viral DNA synthesis, however, that generates duplex-linear DNA (DL-DNA). Deletions of HBV sequences indispensable for the formation of RC-DNA may increase the synthesis of DL-DNA [1]. Both open circular and linear HBV DNA molecules, generated in replication complexes recycled to the nucleus, may initiate integration events that frequently result in deletions or rearrangements both in the viral genome and the invaded cellular DNA [44]. It was suggested that HBV integration events may potentially contribute to oncogenesis by altering the activity of targeted host genes [45]. In a recent massively parallel sequencing study of HCC samples, HBV integration was more frequent in the tumors (86.4%) compared to adjacent liver tissues (30.7%). Within the HBV genome, most of the breakpoints were located in a region comprising the viral enhancer, the HBx gene encoding the regulatory protein wtHBx and the core gene [46]. Deletion of the 3' HBx sequences is a regular consequence of integration [47] and results in the expression of C-terminal truncated HBx (trHBx) proteins that differ in their properties from the full length wtHBx protein. One may speculate that the cellular repair enzymes involved in the RC to cccDNA transition [2,4] may play a role in the generation of HBx deletions. As a matter of fact, a triple helix region in the relaxed, nicked-circular DNA of the HBV genome may facilitate integration into the cellular DNA [48]. Corresponding to this region on the viral genome, virus-cell junctions of integrated viruses clustered just upstream of the expected right-hand end of the DL-DNA at nt 1832. Thus, viral breakpoints mostly locate to the 3'-end of the HBx gene [45,49].

HBV integration into the human genome occurs at the early steps of hepatocarcinogenesis and seems to be a random event [50]. Still, HBV integration at fragile sites of the human genome may alter the expression of close-by oncogenes, tumor suppressor genes and miRNA genes and promote, at least to some extent, tumorigenesis (reviewed by [50]). HBV integration brings about multiple mutagenic events, like deletions, translocations and gross chromosomal abnormalities. Compared to non-viral or HCV-related HCC, the rate of chromosomal aberrations is significantly increased in HBV-induced HCC. Thus, viral insertion may

initiate specific oncogenetic pathways. HBV-driven mutational activity combined with the increased cell turnover in chronic hepatitis constitutes a constantly operating selection system for better than average growing cells [51]. Indeed, the expansion of normal appearing cell clones occurred in tumor-adjacent normal tissue from HBV-HCC patients. Those clones were not restricted to cirrhotic nodules, but occurred also in normal liver tissue and comprised at least 1%, possibly up to 50% of the entire liver tissue [52]. Presumably, those clones of up to thousands of hepatocytes exhibit an as yet unknown selective advantage that drives their expansion within infected livers [49]. It is important to distinguish between an initial, possibly tumor-promoting viral integration event and later genomic rearrangements which accompany tumor progression and may even lead to the loss of the initially integrated viral genome in a hit-and-run fashion. In a cirrhotic liver the HBV genome was lost with the progression of genetic instability from a group of clonally and spatially related cirrhotic nodules [53], (reviewed by [54]).

HBX: A VIRAL PROTO-ONCOGENE EVOLVING IN THE COURSE OF MULTISTEP HEPATOCARCINOGENESIS CODES FOR A MULTIFUNCTIONAL REGULATORY PROTEIN

HBV-associated HCCs regularly contain integrated HBV genomes expressing transcripts for the pleiotropic regulatory protein HBx with a relative molecular mass of 17 kD [10]. Full length HBx, designated as wtHBx in this review, consists of 154 amino acid residues organized into several functional domains [55,56] (Figure 2). The conserved N-terminal "A" region (amino acids 1–20) displayed a transrepressor activity: when coexpressed with wtHBx, it inhibited wtHBx-mediated transactivation in cultured hepatoma cells [55,57]. A proline and serine-rich region (region "B", amino acids 31–57) separates the transrepressor domain from a larger, complex C-terminal region involved in transactivation (residues 58–140) that contains a basic domain (BD) and a cellular protein binding domain (CPBD) [55,56]. The C-terminal region (residues 79–117) of wtHBx promoted an efficient IgG antibody response both in chronic hepatitis patients and in symptomless carriers [58]. The C-terminal region was indispensable for wtHBx stability and contributed to the wtHBx-mediated stimulation of

Hepatitis B virus: HBx-induced patho-epigenetics

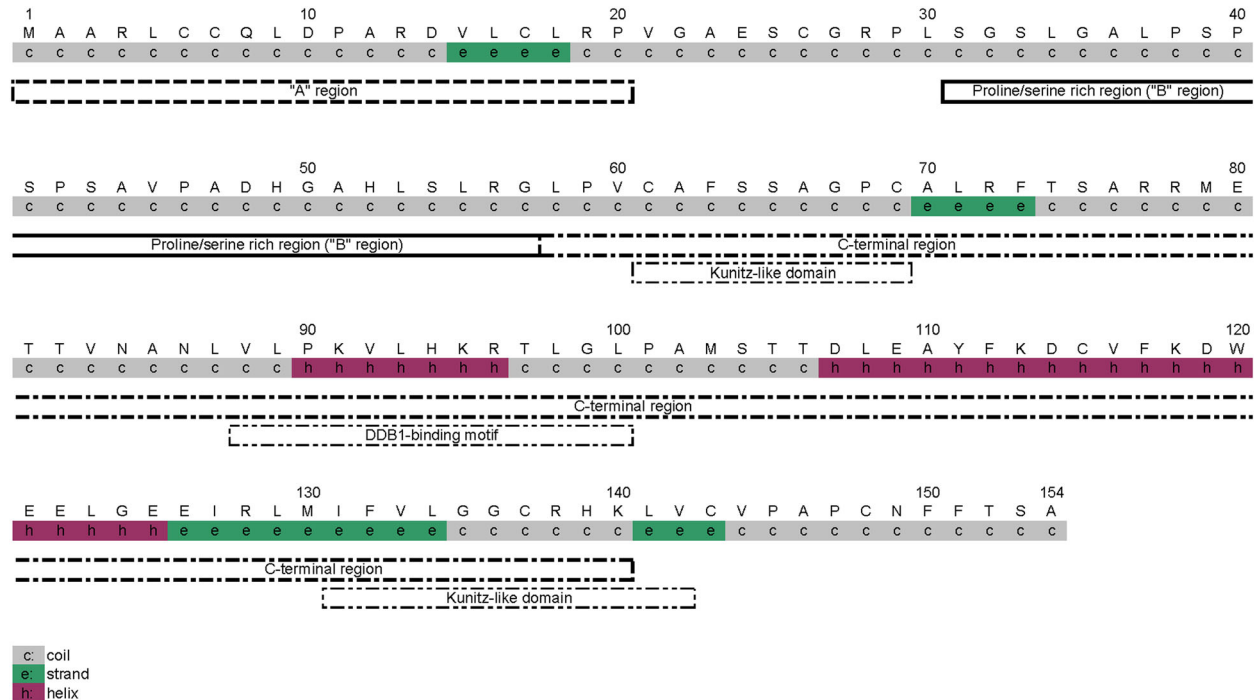


Figure 2. Secondary structure of HBx, genotype C. The conserved N-terminal “A” region (amino acids 1–20) is separated by a proline and serine-rich region (region “B”, amino acids 31–57) from a larger, complex C-terminal region involved in transactivation (residues 58–140) that contains a basic domain (BD) and a cellular protein binding domain (CPBD). The C-terminal region stimulates HBV replication and overlaps with a split Kunitz-like serine protease inhibitor domain (amino acids 61–69 and 131–142) interacting with proteasome subunits. The α -helical DDB1-binding motif (amino acids 88–100) interacts with damaged DNA binding protein 1 (DDB1) that serves as a docking platform for substrate receptor proteins in E3 ubiquitin ligase complexes. The amino acid sequence of HBx, HBV genotype C, was retrieved from the Hepatitis B Virus Database (<https://hbvdb.ibcp.fr/HBVdb/HBVdbIndex>) and was analyzed using the PSIPRED server (<http://bioinf.cs.ucl.ac.uk/psipred/>) as described in [69,70]

HBV replication [59]. This region overlaps with a split Kunitz-like serine protease inhibitor domain (amino acids 61–69 and 131–142) interacting with proteasome subunits. A nine-amino-acid sequence (residues 132–140) from the Kunitz domain induced growth arrest and apoptosis of HepG2 hepatoma cells and down-regulated the expression of p97, an ATPase involved in the ubiquitin proteasome protein degradation pathway [60]. A promiscuous α -helical motif adopted by amino acids 88–100 of wtHBx fits into a large pocket of damaged DNA binding protein 1 (DDB1) that serves as a docking platform for substrate receptor proteins in E3 ubiquitin ligase complexes [61]. This interaction may redirect the ubiquitin ligase machinery to a new, unknown substrate, possibly a cellular protein involved in the antiviral response of the host cell [61]. The ubiquitin ligase complex including DDB1 is involved in the proteolysis of Cdc10-dependent transcript 1 (CDT1), a replication licensing protein rapidly degraded in S phase to prevent

inappropriate origin firing [62–64]. CDT1 is degraded after DNA damage, a mechanism guarding genomic integrity [63]. Thus, interaction of wtHBx with DDB1 may affect the integrity of the genome by modulating CDT1 degradation. wtHBx also interacts with single stranded DNA and a series of DNA repair proteins, resulting in inefficient repair of damaged cellular DNA [65–68]. By blocking DNA repair, wtHBx may facilitate the fixation of mutations and play a role in hepatocarcinogenesis.

In silico modeling of the tertiary structure of wtHBx revealed structural similarity with the central domain of mismatch uracil DNA glycosylase (MUG) enzymes involved in base-excision repair [71]. Because the hypothetical, restored translation product of a vestigial protein X reading frame of duck hepatitis virus showed a similar 3D structure, van Hemert, *et al.* speculated that an ancestral hepadnavirus possibly captured a MUG-like sequence from a host genome [71]. It was also reported that wtHBx is structurally and functionally similar to nucleoside diphosphate

kinases (NDPKs). wtHBx hydrolyzed ATP, transferred a phosphate group to NDP molecules, phosphorylated AMP to ADP and hydrolyzed GTP [72,73]. On the other hand, wtHBx was found to be an intrinsically unstructured protein through several methods, e.g. NMR spectroscopy, circular dichroism spectropolarimetry and bioinformatics. The intrinsic disorder may explain the capability of wtHBx to promiscuously interact with a multitude of target proteins [74].

It is important to note that the various HBV genotypes (A–J) that differ in geographical distribution are different as to their pathogenicity as well [75–77]. Infection with HBV genotypes C and D were linked to a more severe course of liver disease [76]. In addition, genotype C, the most prevalent genotype in Asia, was highly associated with HCC [78]. A recent comprehensive analysis found sequence differences at distinct positions of the HBx gene coding for wtHBx in various HBV genotypes [77]. It is remarkable that in HBV genotype C there were nucleotide differences (mutations) at seven positions between HCC and non-HCC patients. Some of these mutations were present in other HBV genotypes, too [77]. We note, however, that in HuH-7 cells the HBV genotype did not influence the apoptosis-inducing capacity of wtHBx [79].

TRANSCRIPTIONAL ACTIVATION OF HOST GENES BY WTHBX

By interacting with key TFs, wtHBx influences host gene transcription in a pleiotropic manner facilitating liver carcinogenesis. wtHBx-dependent transcriptional activation of the cylooxygenase 2 (COX2) promoter, as verified in HepG2 hepatoma cells, led to elevated prostaglandin E2 (PGE2) levels in chronic HBV patients, thus contributing to liver fibrosis [80]. Transcription of several oncogenes and SREBP-1a, encoding sterol regulatory element binding protein-1a, a key regulator for fatty acid and cholesterol synthesis, was also induced by wtHBx in cultured hepatoma cell lines [81–83]. In Huh-7 hepatoma cells, wtHBx activated FoxM1 encoding forkhead box protein M1, a master regulator of tumor metastasis via the ERK/CREB signaling pathway [84]. wtHBx, via NF- κ B induction, also upregulated a cytokine in HBV carriers and metastasis-associated protein 1 (MTA1), a master chromatin modifier, in HCCs [85,86]. Another important TF which responded to wtHBx expression

in cultured AML12 murine hepatocytes is E2F1, a major regulator of cell cycle-dependent genes [87].

A ChIP-chip approach utilizing anti-HBx antibodies combined with transcriptional profiling on HepG2 hepatoma cells identified 184 gene targets directly regulated by wtHBx, via the interaction with 144 TFs. Among the six signaling pathways affected by wtHBx, the Jak-STAT pathway was the most prominent [88].

wtHBX AND trHBX TRANSGENIC MOUSE MODELS

The pleiotropic actions of wtHBx are also reflected in transgenic mouse models. Transgenic mice carrying the wtHBx gene under the control of the HBV enhancer 1 developed progressive histopathological changes, starting with multifocal areas of altered hepatocytes, later developing benign adenomas and ending in malignant HCCs. Male mice developed HCC much earlier, reflecting the skewed male-pronounced gender ratio of HCC in humans [89]. Another mouse line with a similar transgenic DNA construct yielded tumors after prolonged latency, or biliary duct cysts that possibly corresponded to degenerated tumor tissue [90,91]. Koike, *et al.* observed that low-level wtHBx-expression did not lead to HCC in HBx-transgenic mice, whereas high-level long-term wtHBx-expression led to HCC, preferentially in males [92]. Other wtHBx-transgenic mouse lines did not yield HCC at all [93,94]. A mouse line expressing wtHBx under the control of a liver-specific cellular promoter showed only minor histologic alterations. However, the liver carcinogen diethylnitrosamine (DEN) and wtHBx acted synergistically in the induction of HCC in those mice [95–98]. Carcinogenic synergism was also observed when DEN was replaced by aflatoxin B1 [99]. Cross-breeding of c-Myc transgenic mice with wtHBx-transgenic mice also accelerated tumorigenesis [100,101].

Terradillos, *et al.* found that transgenic expression of wtHBx enhanced apoptotic death in the mouse liver. They speculated that subsequent cycles of cell killing and liver regeneration may select the outgrowth of cell populations resistant to the proapoptotic activity of wtHBx, partly mediated by stimulating the c-myc promoter [102]. Such hepatocyte populations may progress, thereafter, to HCC. In other transgenic models wtHBx inhibited the regeneration of liver tissue in a paracrine manner or led to a premature cell cycle entry

[103–106]. In addition, wtHBx increased the resistance of hepatocytes to reactive oxygen species via upregulation of forkhead protein FoxO4 [107]. Quetier, *et al.* generated transgenic mice expressing either wtHBx or trHBx [108]. They could not observe HCC development in either group. Treatment of young transgenic mice with the liver carcinogen DEN resulted, however, in the development of liver carcinomas. There was a more rapid onset of HCC in the animals expressing trHBx than in the wtHBx transgenic mice [108]. Overall, the transgene experiments showed that the efficiency of integrated HBx in causing HCC in mice is variable and depends on multiple factors. For this reason the HBx gene encoding wtHBx is not generally acknowledged as a classical viral oncogene, although it can induce hepatocarcinogenesis in certain HBx-transgenic strains or may act as a tumor promoter and cofactor.

ABROGATION OF THE ANTI-PROLIFERATIVE AND APOPTOTIC EFFECTS OF wtHBX IN trHBX VARIANTS DEFECTIVE IN TRANSACTIVATION

wtHBx has been reported to either block [109–112] or induce [112–117] apoptosis. These apparently discrepant results may be explained through concentration-dependent effects during different stages of infection [9,118]. Alternatively, HBx mutations may alter the apoptosis-related properties of HBx proteins as well. Expression of wtHBx inhibited colony formation of cells *in vitro* and induced apoptosis by a p53-independent pathway in a set of different cell lines. In contrast, HBx-mutants derived from HCCs including a mutant sequence with both point mutations and a C-terminal truncation resulting in the loss of 26 C-terminal amino acid residues did not interfere with cell growth and lost their transactivation properties, suggesting that the transactivating functions of HBx may be irrelevant to oncogenesis [56].

Further, C-terminal truncations of HBx may explain why HBx was found to induce both apoptosis and anti-apoptosis. In rat embryo fibroblasts, wtHBx acted as a growth-suppressive and proapoptotic protein that blocked the transforming activity of cellular proto-oncogenes or viral oncoproteins [119]. Similarly, Tu, *et al.* observed that in contrast to wtHBx, most of the trHBx proteins obtained from HCCs failed to block cell proliferation and oncogene-mediated transformation of primary rat embryo fibroblasts *in vitro* [119].

The proapoptotic function of wtHBx was mapped to the C-terminal transactivation domain [120]. We note, however, that the situation is complex, because depending on the cell type studied and on the actual experimental conditions, wtHBx was either capable to induce apoptosis or to elicit an anti-apoptotic response in a context-dependent manner (reviewed by [121]).

wtHBx and trHBx differ as to their intracellular localization. In transfected cells, wtHBx accumulated in the cytoplasm and associated with the mitochondria [122–124]. In human liver biopsies derived from patients with chronic HBV infection the localization was also predominantly cytoplasmic, although exclusively nuclear or cytoplasmic and nuclear localization was also observed [125]. trHBx expressing transfected HepG2 cells displayed, however, a predominant nuclear localization [124]. Because wtHBx facilitates the translocation of Bax, a proapoptotic protein, to mitochondria and causes fragmentation and swelling of the organelle [122,126,127], one may speculate that the lack of proapoptotic function of trHBx is partly because of its altered cellular localization.

Integration of HBV DNA into the cellular genome is not required for viral replication. Still, HBV tends towards multiple integrations during chronic infection. Integrated HBV sequences were observed in 80–90% of HCC cases [46,128], (reviewed by [50,129]). Viral integrants isolated from HCCs contained trHBx genes coding for C-terminal trHBx proteins unable to transactivate and unable to suppress cell proliferation [119]. Instead of suppressing the transforming functions of oncogenes, like wtHBx proteins did, the trHBx proteins enhanced the transforming abilities of those oncogenes [119].

Sequencing of 14 viral integration sites in HCCs revealed rearrangements of the viral genome in all cases; in 10 of 14 cases HBx was truncated at the C-terminus [130]. Using immunocytochemistry with antibodies detecting either full length HBx only or both full length and trHBx proteins, Ma, *et al.* detected trHBx in 88 out of 111 HCC tissue samples (79%), whereas wtHBx was present in 23 of 111 tumorous tissues (20.7%) [47]. In cultured hepatocytes, expression of trHBx, but not wtHBx transformed the immortalized cell line MIHA [47]. Sze, *et al.* found trHBx mRNA and protein, respectively, in 23 and 22 out of 50 HCCs; wtHBx only occurred in the tumor-surrounding liver tissue, where trHBx was absent. Expression of trHBX

facilitated invasive growth, matrix metalloproteinase 10 (MMP10) expression and angiogenesis [131]. Furthermore, a comprehensive analysis of a set of more than one thousand integrated HBV sequences from diverse genotypes and disease stages showed that HCC-associated mutations clustered within the C-terminal end of the HBx gene, especially for genotype C. Some of these mutations preexisted already at pretumoral disease stages [77].

Analysis of microRNA transcription in cells expressing wtHBx or trHBx showed that hepatocytes expressing trHBx grew faster and showed a decreased expression of a set of growth-suppressive microRNAs, while wtHBx increased the expression of that miRNA set [132]. Thus, C-terminal truncation of wtHBx seems to be important for the development of HCC (Table 1). The deletion may be causal at the beginning of liver tumorigenesis by providing anti-apoptotic functions for naturally apoptosis-bound oncogenic cells, or the growth-suppressive functions of the HBx C-terminus may be selected against at a later stage of tumor development.

wtHBX MAY DRIVE SEQUENTIAL EVENTS OF HEPATOCARCINOGENESIS FROM A CHRONICALLY HBV INFECTED PREMALIGNANT STATE TO METASTATIC HEPATOCELLULAR CARCINOMA

wtHBx induced supernumerary centrosomes and multipolar spindles in cultured cells, and aneuploidy of chromosomes 1 and 6 was detected in HBV infected hepatocytes of chronically HBV infected patients prior to the development of HCC. These events were attributed to the wtHBx-mediated induction of

cytoplasmic sequestration of Crm1, a nuclear export receptor preventing extra centriole synthesis [138]. Thus, wtHBx may initiate hepatocarcinogenesis by eliciting genomic instability and chromosomal aberrations already at a pretumoral stage.

Chronic HBV infection may result in necrosis and rapid regeneration of hepatocytes, accompanied with fibrosis that leads eventually to cirrhosis, the most important precancerous condition predisposing to HCC development [133,139] (Table 1). wtHBx induced transforming growth factor- β (TGF- β) secretion in hepatocytes that activated, in a paracrine manner, the proliferation and gene expression of hepatic stellate cells (HSCs) [139]. Because HSCs are the main producers of extracellular matrix proteins in the fibrotic liver, HBx appears to be an important player in the development of liver cirrhosis.

wtHBx may also utilize a paracrine mechanism to inhibit the proliferation of hepatocytes. This process may select, however, for cells resistant to paracrine inhibition [103]. Thus, wtHBx mutations or truncations abrogating the proapoptotic effect of the protein [119], or changes to the differentiation status of hepatocytes may result in hyperplastic hepatocyte nodules that may progress to dysplastic nodules, the putative precursors of HCC [133]. In the livers of HBx-transgenic mice dysplastic lesions displayed a more intensive staining for wtHBx, hypoxia-inducible factor-1 alpha (HIF1 α) and vascular endothelial growth factor (VEGF) compared to non-neoplastic regions [140]. Abnormally large blood vessels were also detected in the dysplastic lesions where capillary-like microvessels were more abundant than in non-neoplastic regions.

Table 1. Biological effects and properties of wtHBx and trHBx

	Protein		
	wtHBx	trHBx	Reference
Effect/property			
Stimulation of HBV replication	Yes	No	[59]
Induction of apoptosis	Yes	No	[120]
Inhibition of cell growth	Yes	No	[56,119]
Transactivation	Yes	No (?)	[56]
Inhibition of cell transformation	Yes	No	[119,120]
Predominant localization	Cytoplasm	Nucleus	[122–124,127]
Expression in hepatocellular carcinoma cells	Yes	Yes	[47]
Expression in nontumorous tissues	Yes	No	[131]
Enhancement of invasiveness and metastasis formation	Yes	Yes	[131,133–137]

Because wtHBx prevented the degradation and increased the transcriptional activity of HIF1 α , a subunit of the TF HIF-1 that controls angiogenesis and VEGF expression, Moon, *et al.* suggested that wtHBx may promote hepatocarcinogenesis by the induction of angiogenesis already in the dysplastic nodules, i.e. at an early stage of neoplastic development [140].

wtHBx induced the disruption of adherens junctions resulting in the dissociation of the cadherin–cytoskeleton interaction and decreased homotypic adhesion between hepatocytes [141]. Because the loss of cadherin-dependent adhesion facilitates cell migration and invasive behavior, Lara-Pezzi, *et al.* speculated that by disrupting cell–cell adhesion wtHBx may induce the progression from adenoma to carcinoma [141]. wtHBx may also facilitate invasion by increasing cell motility and matrix metalloproteinase-9 (MMP-9) production [133–137]. Upregulation of MMP-1 and cyclooxygenase-2 (COX-2) expression by wtHBx also correlated with enhanced tumor cell invasion [142]. wtHBx expression facilitated the generation of intrahepatic and lung metastases in nude mouse, possibly by inducing epithelial–

mesenchymal transition (EMT), a phenomenon associated with changes of cell morphology and behavior [143–145]. As mentioned above, trHBx enhanced the invasive ability of transfected cells by activating MMP10 expression [131] (Table 2).

We notice here that there are also other scenarios of hepatocarcinogenesis. Although worldwide more than 90% of HCC are based on previous cirrhosis, about 40% of HCC in the southern half of Africa arise without previous cirrhotic lesions (reviewed by [146]). In these patients, chromosomal instability (CIN) appears to be an important factor in HCC development (reviewed by [147,148]).

EPIGENETIC DYSREGULATION IN HBV INFECTED CELLS: THE ROLE OF wtHBX

Epigenetic modifications especially DNA methylation patterns clearly distinguish HBV-associated HCC from HCC of other origins. Alcohol-caused HCC correlated with hypomethylation at O-6-methylguanine-DNMT (MGMT), while glutathione S-transferase P (GSTP1) was hypermethylated in HBV-associated HCC [149]. Methylation at the kallikrein-10 (KLK10) promoter correlated with

Table 2. The role of wtHBx and trHBx in successive stages of hepatocarcinogenesis

Stage	HBx structure	Effect
Premalignant hepatic lesions	wtHBx	Centrosome abnormalities
Cirrhosis	wtHBx	Paracrine stimulation, proliferation of hepatic stellate cells, collagen deposition Induction of cyclooxygenase-2, increased PGE2 production, fibrosis Induction of hepatocyte apoptosis Paracrine stimulation, inhibition of liver cell proliferation Selection of liver cells carrying deleted HBx genes Selection of apoptosis-resistant, proliferating liver cells
Hyperplastic nodule	wtHBx, mutated HBx, trHBx (?)	
Dysplastic nodule	wtHBx, mutated HBx, trHBx	Induction of angiogenesis
Adenoma	wtHBx, mutated HBx, trHBx	Loss of homotypic adhesion
Carcinoma	wtHBx, mutated HBx, trHBx	Epithelial–mesenchymal transition
Invasion	wtHBx, mutated HBx, trHBx	Increased motility, production of metalloproteinases
Metastasis	wtHBx, mutated HBx, trHBx	Increased motility, production of metalloproteinases

HCV-associated cirrhosis and HCC, but correlated inversely with HBV [150]. Recent methylome analyses supported these early observations and revealed the presence of both etiology-specific and shared DNA methylation alterations in HCCs [149,151].

The mechanisms involved in the alteration of host cell methylation patterns in HBV infected cells remain to be clarified. Vivekanandan, *et al.* suggested that HBV infection may induce DNMTs that methylate and silence the viral genome and act as effectors of an antiviral defense mechanism [34] (see also "Epigenetic regulation of the HBV genome"). Hypermethylation of distinct cellular promoters may be a byproduct of such a defense mechanism. Another possibility is that one or several HBV proteins expressed in the host cells interact with the cellular epigenetic machinery and cause epigenetic dysregulation. The best candidate for such a role is wtHBx. The role of trHBx in the alteration of the host cell epigenome remains to be established. We note however, that the epigenetic alterations induced by wtHBx in hepatocytes during the initial phase of carcinogenesis may persist at a later stage, after the appearance of trHBx as well (epigenetic memory). The role of trHBx in epigenetic dysregulation remains to be established.

In HBV-associated HCCs distinct epigenetic changes occurred already at early stages of carcinogenesis. The tumor suppressor genes APC, RASSF1A and SOCS1 were methylated in a fraction of HBV-associated cirrhotic nodules. The methylation levels of APC and RASSF1 increased further in low-grade dysplastic nodules, whereas SOCS1-methylation peaked in early HCC, and decreased in advanced liver carcinomas [152].

Methylation profiles based on the analysis at 27000 CpG-dinucleotides within 14495 promoters of single hepatocytes from HBV-associated HCC tumor tissue distinguished malignant, tumor-adjacent and normal hepatocytes [153]. A set of seven genes were hypermethylated in malignant, but hypomethylated in tumor-adjacent hepatocytes, whereas a distinct group of nine genes was hypermethylated both in malignant and non-malignant tumor-adjacent hepatocytes. The latter data supported the epigenetic field theory for cancerization [154], and other observations of an epigenetic field during HCC development [155].

Hypermethylation at the CGIs of p16INK4A and CCND2 was increased with progredient tissue

damage from normal tissue via cirrhosis to HCC, indicating that hypermethylation of the p16 locus may prepare the ground for hepatocarcinogenesis [156]. The promoter of sFRP1, a gene coding for secreted frizzled-related protein 1 that blocks Wnt signaling, was significantly more often silenced by hypermethylation in HCC than in surrounding tissue and may be an early marker of HCC [157]. E-cadherin is a key cellular adhesion protein which acts as a tumor suppressor. Its absence leads to a more invasive growth and metastasizing behavior of tumor cells. In preneoplastic tissue and early stages of HCC, the E-cadherin (CDH1) promoter was silenced by DNA methylation [158–161]. wtHBx induced transcriptional repression of CDH1 via activation of DNMT1 and increased cell migration [162,163]. In addition to increased promoter methylation, build-up of a complex of repressor proteins also contributed to the silencing of E-cadherin transcription. wtHBx recruited the corepressor protein mSin3A, which is the core of an HDAC complex, to the CDH1 promoter [164].

Premature senescence which acts as a tumor suppressing mechanism through growth arrest in G1 was blocked in HepG2 cells by wtHBx-mediated hypermethylation at p16 [165]. DNMT1 induction possibly occurred via the p16INK4A-cyclin D1-CDK4/6-pRb-E2F1 pathway [166–168]. In addition to the upregulation of DNMT1 expression, wtHBx also induced two splice variants of the de novo DNMT DNMT3A (DNMT3A1 and DNMT3A2) in hepatoma cell lines resulting in hypermethylation of the tumor suppressor gene IGFBP3 via recruitment of DNMTs and MeCP2 to the IGFBP3 promoter [169]. wtHBx silenced additional cellular promoters as well by recruiting DNMT3A2 or upregulating DNMT1 and DNMT3A [170–173]. In contrast, wtHBx downregulated DNMT3B, the other de novo DNMT. Because DNMT3B is involved in the methylation of satellite 2 repeats, those sequences were hypomethylated [169]. It is worthy to note that LINE1 repetitive sequences were found to be hypomethylated in the sera from HCC patients and hypomethylation correlated with HBs carrier status, large tumor sizes and poor survival [174]. The same methylation patterns, methylation at IGFBP3 and global hypomethylation at repetitive sequences were found in HCC biopsies from 20 patients. Thus, HBV infection may accelerate hepatocarcinogenesis by epigenetic means right after the beginning of HBV infection

[169]. In addition to promoter hypermethylation, other mechanisms are also involved in wtHBx-induced repression of cellular genes, including wtHBx/HDAC1 complex formation and deacetylation of the TF Sp1 [170,175]. Gene activation by wtHBx was also observed, via removal of DNMT3A from cellular promoters or induction of promoter hypomethylation [170,176].

Lee, *et al.* compared the methylomes of hepatocytes from normal and wtHBx-transgenic mice using the methylated CpG island recovery assay (MIRA) [177]. They observed a decrease in global DNA methylation both in 3-month-old and 13-month-old wtHBx-transgenic mice with HCC. In addition to the demethylation of the satellite elements, intragenic CpG islands overlapping the distal exons were also frequently hypomethylated in HBx-induced liver tumors [177]. It is worthy to note, that in contrast to promoter-associated CpG islands where hypomethylation typically correlates with promoter activity, a reduction of transcript levels was observed at the hypomethylated intragenic CpG islands. Lee, *et al.* argued that wtHBx-mediated downregulation of the murine Dnmt3a and its partner, Dnmt3l, resulted in hypomethylation of intragenic CpG islands. wtHBx bound to the major Dnmt3l promoter in wtHBx-transgenic mouse hepatocytes and possibly silenced it via the recruitment of HDAC1 [177]. Further studies may clarify the inconsistencies between the *in vitro* and *in vivo* data regarding the effect of wtHBx on human DNMT3A and murine Dnmt3a levels, respectively [169,177]. It also remains to be established how the methylation patterns of intragenic CpG islands change in HBV infected human cells.

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CONCLUSION

Based on the fundamental differences between the effects of wtHBx and trHBx on cell physiology and the regular appearance of trHBx during HCC development we suggest that HBx acts similarly to a proto-onc gene acquired during evolution by the HBV genome. Only the full-length HBx gene, coding for a proapoptotic, “wild type” HBx protein (wtHBx) is transmitted during the viral replication cycle.

Expression of wtHBx in infected hepatocytes induces genetic and epigenetic alterations and may elicit necrosis and rapid compensatory proliferation of hepatocytes, accompanied with fibrosis that leads eventually to cirrhosis, the most important precancerous condition predisposing to HCC development. In parallel, in distinct hepatocytes of newly infected individuals HBx is activated by deletion, in analogy to some retroviral v-onc genes resulting in a viral oncogene, that codes for an antiapoptotic, C-terminal trHBx protein that stimulates cell proliferation and facilitates development and progression of HCC. In the hepatocytes of HCC patients, coexpression of wtHBx and trHBx was demonstrated in distinct hepatocyte populations. HCC typically develops, however, from proliferating hepatocytes carrying deleted HBV genomes coding for trHBx.

CONFLICT OF INTEREST

The authors have no competing interest.

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