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Hepatitis B Virus X and Regulation of Viral Gene Expression

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The efficient replication of hepatitis B virus (HBV) requires the HBV regulatory hepatitis B virus X (HBx) protein. The exact contributions of HBx are not fully understood, in part because of the limitations of the assays used for its study. When HBV replication is driven from a plasmid DNA, the contribution of HBx is modest. However, there is an absolute requirement for HBx in assays that recapitulate the infectious virus life cycle. There is much evidence that HBx can contribute directly to HBV replication by acting on viral promoters embedded within protein coding sequences. In addition, HBx may also contribute indirectly by modulating cellular pathways to benefit virus replication. Understanding the mechanism(s) of HBx action during virus replication may provide insight into novel ways to disrupt chronic HBV replication.

The replication efficiency of viruses, such as the human hepatitis B virus (HBV), is facilitated by the regulatory proteins they encode. This is particularly important for viruses that infect differentiated cells, such as hepatocytes, which are typically quiescent and may not present an ideal intracellular environment for replication of a DNA virus. Although viruses with large amounts of genetic information may encode multiple regulatory proteins, the 3.2-kb HBV genome encodes a single regulatory protein called hepatitis B virus X (HBx) (Fig. 1) (reviewed in Seeger et al. 2013). All mammalian hepadnaviruses encode an X protein, but the avian hepadnaviruses do not. HBx resides in the cytosol and nucleus of HBV-infected cells where it can modulate numerous cellular signal-transduction pathways and interact with various cellular proteins (Fig. 2) (reviewed in Bouchard and Schneider 2004; Neuveut et al. 2010; Wei et al. 2010b). Efforts to define the functions of HBx during HBV replication are ongoing. However, HBx has been difficult to study because of limitations of available assays, including the inability of HBV to infect most cells in culture, the HBV genome structure of overlapping open reading frames, and the difficulties in working with the 17-kDa HBx protein for which few antibody reagents are available. Nevertheless, there is an abundance of data indicating that HBx enhances HBV replication, likely through both direct and indirect mechanisms, and usually by cooperating with the cellular signal-transduction machinery (reviewed in Bou-

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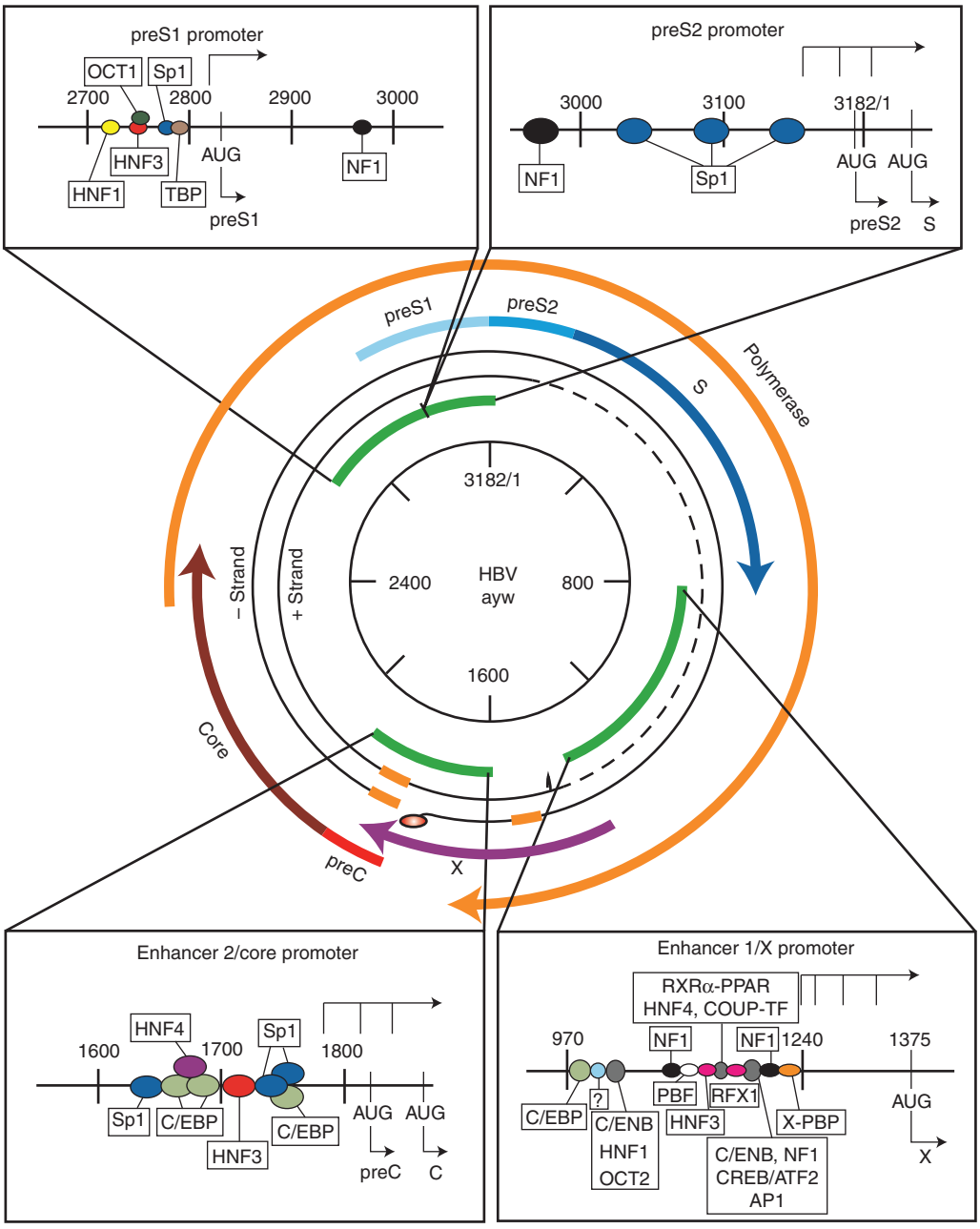

Figure 1. Hepatitis B virus (HBV) genome organization. The HBV genome contains four overlapping open reading frames (ORFs) depicted by the colored arrows. The X ORF (purple) encodes the hepatitis B virus X (HBx) regulatory protein. Boxes contain viral regulatory elements: viral promoters (preS2, preS1, Core, and X) and enhancer elements (ENHI and ENHII), with their position in the genome indicated by green bars. Multiple liver-specific and ubiquitous transcription factors bind to HBV regulatory elements (see text for a detailed description, including abbreviations).

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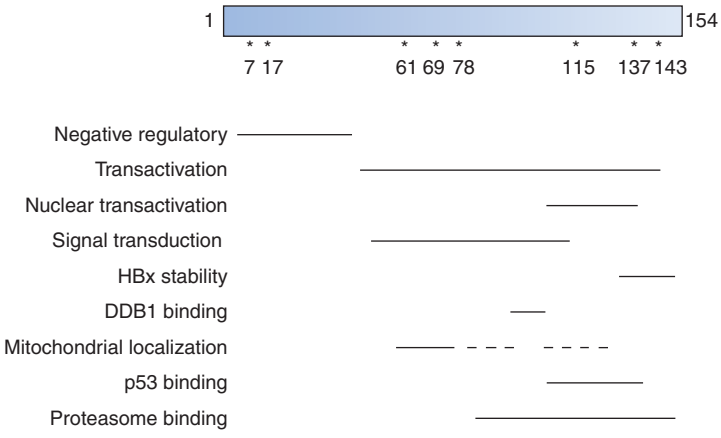

Figure 2. Domains of hepatitis B virus X (HBx). The 154-amino-acid (aa) HBx protein is shown, with the asterisks indicating the location of conserved cysteines. Lines below the HBx protein indicate the domain of HBx that retains the function listed at the *left*, as described in the text.

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chard and Schneider 2004; Neuveut et al. 2010; Wei et al. 2010b). Most studies that have investigated the function of HBx in the context of HBV replication have compared viral markers of replication (DNA, RNA, or protein) in liver cells transiently transfected with a plasmid DNA encoding a greater-than-unit length HBV genome capable of expressing HBx (Scaglioni et al. 1997) to the same plasmid DNA that contains a point mutation preventing the expression of HBx (Melegari et al. 2005). Recently, these approaches have been expanded to studies in cultured primary hepatocytes and in the livers of mice with normal or humanized livers (Clippinger and Bouchard 2008; Clippinger et al. 2009; Gearhart and Bouchard 2010a,b; Tsuge et al. 2010). Cumulatively, these studies have yielded important information on HBx functions in the context of HBV replication. Continuing studies to define HBx functions during HBV replication are likely to contribute to the ongoing efforts to design novel therapeutic strategies to interrupt HBV replication and prevent the development of HBV-associated liver diseases.

REQUIREMENT OF HBx IN VIRUS REPLICATION

The first experiments to investigate a role for HBx in HBV replication were conducted in plasmidtransfected human liver HuH-7 cells. No significant differences were found in levels of viral proteins, RNA or DNA, when comparing cells transfected with plasmid DNA carrying HBV genomes encoding HBx versus genomes with a mutation that prevented HBx expression, and it was concluded that HBx was not central to the virus life cycle in vitro (Blum et al. 1992). A similar approach using human hepatoblastoma HepG2 cells showed that HBV genomes encoding HBx replicated to significantly higher levels than genomes unable to express HBx (Melegari et al. 1998), and that cotransfected plasmids encoding HBx could restore the HBx-deficient replication to wild-type levels in these cells (Bouchard et al. 2002; Leupin et al. 2005; Tang et al. 2005; Keasler et al. 2007). Interestingly, the effect of HBx is most apparent in HepG2 cells that are quiescent (Bouchard et al. 2002; Leupin et al. 2005; Keasler et al. 2007), a cellular environment similar to the nondividing hepatocyte in vivo. These studies show that HBV can be produced from a plasmid DNA template in transfected cells in the absence of HBx, and that the level of virus replication is increased in the presence of HBx. Similar observations were recently reported in cultured primary rat hepatocytes infected with a recombinant adenovirus containing a wild-type or HBx-deficient copy of the HBV genome (Clippinger et al. 2009).

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Independent animal models similarly conclude a role for HBx in HBV replication in vivo. Mice harboring the HBV genome with an inactivating mutation that prevents HBx expression produced virus particles (Reifenberg et al. 2002; Xu et al. 2002), but the levels of virus were increased by crossbreeding with mice that expressed HBx (Xu et al. 2002). Hydrodynamic tail vein injection was used to deliver the HBV and HBx-deficient HBV plasmid DNA described above to mouse liver in vivo, and in this model HBx deficiency resulted in a 2-log decrease in viremia that was restored to wild-type levels by coinjection of plasmid DNA encoding HBx (Keasler et al. 2007). Intrahepatic injection of plasmid DNA encoding woodchuck hepatitis B virus (WHV) DNA containing mutations in WHx similarly led to lower levels of replication than wild-type WHV DNA (Zhang et al. 2001). Together, these studies indicate that HBV replication driven from a plasmid template in vivo can occur in the absence of HBx, but that HBx boosts the efficiency of that replication.

The most compelling evidence that HBx is critical for the virus life cycle comes from two animal models that more closely recapitulate the HBV life cycle. In the first animal model, intrahepatic inoculation of woodchuck hepatitis virus (WHV) genomes capable of expressing woodchuck X protein (WHx) led to replication in woodchuck hosts, whereas WHx-deficient genomes did not replicate and the animals remained susceptible to subsequent viral challenge (Chen et al. 1993; Zoulim et al. 1994). In the second animal model, immunodeficient mice transplanted with human hepatocytes can be infected with HBV (Dandri et al. 2001; Bissig et al. 2010). Virus generated by transfection of HepG2 cells with a plasmid HBV DNA encoding HBx or a plasmid HBV DNA containing an HBx mutation that prevented HBx expression were used to infect human-liver chimeric mice (Tsuge et al. 2010). Although HBx-proficient HBV particles established viremia in these animals, the HBx-deficient virus did not (Tsuge et al. 2010). Hydrodynamic injection of these mice with HBx-expression plasmid restored the replication of the HBx-deficient virus. This absolute requirement of HBx for HBV replication was also confirmed in the human HepaRG cell line, which can be directly infected by HBV, and in cultured human hepatocytes (Lucifora et al. 2011). In summary, the requirement for HBx for HBV replication is most convincing in model systems that recapitulate the virus life cycle in vivo. In contrast, the impact of HBx on HBV replication is modest when virus replication is driven from a plasmid DNA template transfected into established cell lines, which could reflect the constitutive activation of various cellular-signaling pathways in these cell lines that HBx must activate in normal hepatocytes in vivo (reviewed in Bouchard and Schneider 2004; Seeger et al. 2007).

HBx PROTEIN STRUCTURE

When the HBV genome was first sequenced and revealed the existence of a novel small open reading frame (ORF), the HBV protein encoded in this ORF was given the name X protein, or HBx, because its sequence had no homology with known protein motifs that might provide clues to its function (reviewed in Tiollais et al. 1981). Early attempts to define the structure of HBx and relate HBx structure to its functions relied on a combination of prediction models and activity studies of mutant HBx proteins (reviewed in Yen 1996). Additional approaches included purification of HBx from bacterial or insect cell expression systems and analyses of posttranslational modifications of HBx, such as acetylation, phosphorylation, and the formation of disulfide bonds (Lin and Lo 1989; Schek et al. 1991; Urban et al. 1997).

Studies of HBx deletion mutants have identified domains of HBx responsible for its function (Fig. 3). The transactivation functions of HBx (described below) reside between amino acid (aa) 52–148, whereas aa 1–50 encode a domain that can inhibit HBx activities (Murakami et al. 1994). Further studies defined aa 120–140 as involved in nuclear transactivation mechanisms, aa 58–119 as involved in signal-transduction activities, and the carboxy-terminal 20 aa as involved in HBx stability (Lizzano et al. 2011). HBx aa 54–70 were later

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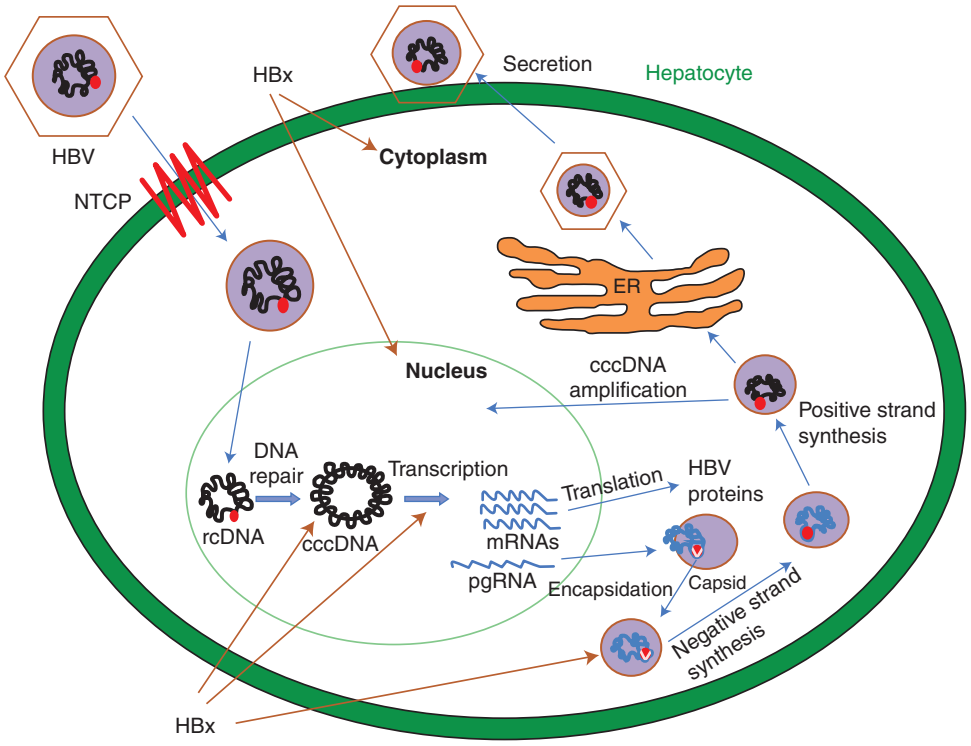

Figure 3. Hepatitis B virus (HBV) life cycle and hepatitis B virus X (HBx). HBV infects hepatocyte by first interacting with its newly identified cell-surface receptor, the sodium-taurocholate cotransporting polypeptide (NTCP; also referred to as SLC10A1) (Yan et al. 2012, 2013; Zhong et al. 2013). The HBV partially double-stranded DNA genome is delivered to the nucleus and converted to a covalently closed circular DNA (cccDNA), which forms a minichromosome and is the source of HBV transcripts. The transcripts are exported to the cytosol for translation, and one of the transcripts, the pregenomic RNA is encapsidated and reversed transcribed to generate the viral DNA genome. Infectious virions are eventually secreted from the cell. HBx is localized to the cytoplasm and nucleus of HBV-infected cells and regulates various stages of HBV replication (arrows). It stimulates HBV transcription both by activating cellular transcription pathways and by affecting epigenetic signals on the cccDNA minichromosome. HBx regulation of cellular signal-transduction pathways also stimulates the HBV reverse transcriptase to facilitate generation of the DNA genome (reviewed in Seeger et al. 2007). ER, Endoplasmic reticulum; rcDNA, relaxed circular DNA; mRNA, messenger RNA; pgRNA, pregenomic RNA.

shown to be essential for HBx localization to mitochondria, whereas aa 75–88 and 109–131 were shown to aid in the localization of HBx to mitochondria (reviewed in Kumar and Sarkar 2004; Wei et al. 2010b). HBx aa 82–154 are sufficient to mediate binding to proteasome subunits (Zhang et al. 2000), whereas p53 binding is mediated by aa 102–136 (Lin et al. 1997b). It is important to note, however, that without a defined 3D structure of HBx as a basis for studying mutant HBx proteins, it remains unclear whether observed effects of the mutant HBx proteins that were analyzed reflected identification of regions of HBx, which are required for specific functions or were caused by disrupting the overall structure of HBx.

Analyses of posttranslational modifications of HBx have identified acetylation, phosphorylation, and disulfide bond formation as potential modifications of HBx, and recent predictive modeling studies have identified putative *O*-linked glycosylation sites (Lin and Lo 1989; Schek et al. 1991; Urban et al. 1997; Hernandez et al. 2012). However, the significance of observed posttranslational modification of HBx for its functions remains unclear. Acetylation

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of HBx has only been observed for HBx that is purified from insect cells (Urban et al. 1997), and, with the exception of a recent observation that AKT phosphorylation of HBx on amino acid residue serine 31 may affect the oncogenic potential of HBx (Khattar et al. 2012), the consequence of HBx phosphorylation during a natural HBV infection remains unclear. Moreover, studies showing disulfide bond formation in samples of purified HBx protein must be interpreted with caution because disulfide bond formation can be an artifact of protein purification methods (Leon et al. 1997; Locker and Griffiths 1999). A majority of HBx is present in the cytosol (Sirma et al. 1998), yet disulfide bond formation in the reducing environment of the cytosol is uncommon (Leon et al. 1997; Locker and Griffiths 1999). Moreover, the results of circular dichroism (CD) spectroscopy studies with a truncated mutant of HBx (containing aa 18–142) in which all cysteines had been deleted or converted to serines suggested that disulfide bond formation between cysteines within the HBx amino acid sequence is not necessary for HBx structure (de Moura et al. 2005; Lee et al. 2012). However, these conclusions should also be interpreted with caution because the assays used to test the functionality of HBx may not represent actual HBx activities during an HBV infection. Overall, attempts to understand HBx structure and function with purified HBx or HBx mutants have identified regions of HBx that appear important for particular HBx activities, but these studies lack clear proof that the mutant proteins retained structures that resemble wild-type HBx.

HBx has been difficult to purify in large quantities and, to date, HBx has not been crystalized. Consequently, with the exception of one domain that was cocrystalized with its interacting partner DDB1 (Li et al. 2010), the overall structure of HBx has been characterized by less precise structural assays, such as CD, fluorescence, and nuclear magnetic resonance (NMR) spectroscopy analyses or predictive in silico bioinformatics approaches (de Moura et al. 2005; van Hemert et al. 2011; Hernandez et al. 2012; Lee et al. 2012). An early attempt to characterize protein secondary structures in HBx predicted the presences of amino- and carboxy-terminal α-helices but otherwise only described patches of hydrophobic and charged residues within the HBx amino acid sequence (Colgrove et al. 1989). Subsequently, studies using a truncated version of HBx (aa 18–142) and analyses by CD, NMR, and predictive bioinformatics modeling programs, suggested that the amino-terminal 30 amino acids of the truncated HBx, or potentially the amino-terminal 50 amino acids of wild-type HBx, are unstructured, whereas the carboxy-terminal portion of HBx is structured (de Moura et al. 2005; Lee et al. 2012). These studies did not define the exact structures present in the carboxy-terminal portion of HBx. More sophisticated computer-modeling programs have predicted HBx structures used for in silico modeling of the overall structure of HBx (van Hemert et al. 2011; Hernandez et al. 2012). One study used this technique to model HBx structure in combination with an analysis of conserved HBx sequences in different HBV isolates that might indicate sites of phosphorylation or modification by *O*-linked glycosylation with *N*-acetylglucosamine (*O*-GlcNac) via a cytosolic glycosylation pathway (Hernandez et al. 2012). The results of this study predicted that there are numerous α-helical structures in the central portion of HBx, that serine 25 and 41 and threonine 81 of HBx are exposed on the surface of HBx, and that these aa residues are contained within conserved phosphorylation or glycosylation sequences. Whether this in silico prediction of phosphorylated and glycosylated sites on HBx accurately reflect in vivo modifications of HBx awaits further confirmative studies. Of note is that the in silico study did not predict phosphorylation of HBx serine 31, which was identified as a site of AKT phosphorylation in another study (Khattar et al. 2012); however, the in silico modeling system focused on HBx aa residues that are conserved in multiple HBV genotypes, and the HBx serine 31 in the previous study is not conserved (Hernandez et al. 2012). In silico modeling was also used to compare the predicted 3D structure of HBx with cellular proteins. This analysis suggested that HBx has considerable structural homology

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with a DNA glycosylase (van Hemert et al. 2011). Support for the accuracy of the structure generated by this in silico modeling method was provided by comparison to a portion of HBx that had been previously cocrystallized with the cellular damaged DNA-binding protein 1 (DDB1) (Li et al. 2010). In this cocrystallization study, a region of HBx was shown to contain a three-turn α-helical structure that permits interaction with DDB1, an important adaptor protein for the Cullin 4 E3 ligase complex. The three-turn α-helical motif is also found in many cellular proteins that bind to the same region of DDB1 to which HBx binds. Importantly, the in silico modeling approach of the 3D structure of HBx and the crystal structure characterized in HBx-DDB1 cocrystals were similar for the region common to both studies. Although in silico docking of HBx to DNA supported the notion that its structure is similar to a DNA glycosylase, HBx does not bind to double-stranded DNA in vivo, and whether it can bind to single-stranded DNA in an authentic HBV infection remains unclear (reviewed in Bouchard and Schneider 2004; Wei et al. 2010b). Consequently, although the in silico generation of a 3D structure of HBx provides a platform for targeted mutagenic studies of HBx to interrogate structure–function relationships, precisely how this putative DNA glycosylase structure is related to HBx functions remains to be determined.

HBx ACTS ON HBV ENHANCERS AND PROMOTERS

The generation of HBV transcripts encoding the viral precore, core, envelope, polymerase, and HBx protein is controlled by four transcription promoters and two enhancers (Fig. 1) (reviewed in Seeger et al. 2013). Transcription factors that bind HBV promoters and enhancers include hepatocyte nuclear factor (HNF)1, HNF3 (Fox A), HNF4α, CCAAT-enhancer-binding protein (C/EBP)1, activating transcription factor (ATF), cAMP response element–binding protein (CREB), SP1, Oct1, and peroxisome proliferator–activated receptor (PPAR)γ (reviewed in Seeger et al. 2007). Enhancer I (ENHI) contains binding sequences for transcription factors that are expressed in many cell types, whereas ENHII contains recognition sequences for hepatocyte-specific transcription factors (reviewed in Seeger et al. 2007). Consequently, the activity level of HBV enhancers and their stimulation of HBV promoters can vary depending on the cell types used to assess the activity of these elements. Interestingly, in vivo studies in mice have shown that the activity of the HBV enhancers and their ability to stimulate transcription from HBV promoters is significantly greater in vivo than in cell lines (Du et al. 2008). In these studies, the activity of each of the four HBV transcription promoters linked to ENHI or ENHII and a luciferase reporter was tested in transfected cells versus following hydrodynamic delivery of the same plasmids to mouse liver in vivo; the in vivo level of luciferase was monitored by bioluminescence imaging and compared to luciferase levels in the transfected Hepa1–6 mouse hepatoma cells. In contrast to the modest strength of these viral regulatory elements in transfected mouse Hepa1–6 cells in vivo, the enhancers stimulated the promoters by 17- to 180-fold (ENHI) and 14- to 140-fold (ENHII) (Du et al. 2008). Importantly, these results also suggest that previous studies that have relied on assays in cell lines may have underestimated HBV protein levels that are present in a natural HBV infection.

Although transcription from HBV promoters and enhancers is not absolutely dependent on HBx expression, HBx can stimulate transcription of both HBV and cellular transcription–regulatory elements in various cell lines and in vivo in hepatocytes (reviewed in Bouchard and Schneider 2004; Gearhart and Bouchard 2010b; Wei et al. 2010b). HBx has sometimes been described as a "weak" transactivator, but this description is inconsistent with the impact of HBx on expression of HBV transcripts and the highly efficient HBV replication observed in vivo. One probable explanation is that liver-enriched transcription factors that are activated by HBx in vivo in differentiated hepatocytes are present at greatly altered levels in dedifferentiated cell lines (Schrem et al. 2002, 2004) that are often used to analyze HBx trans-

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activator functions, and the two- to fourfold transactivation observed for HBx in cell lines may represent only a portion of its function in vivo.

HBx localization to the nucleus and cytosol has been linked to its transcriptional activity, and nuclear or cytosolic HBx can affect different transcription factors (reviewed in Bouchard and Schneider 2004; Benhenda et al. 2009; Wei et al. 2010b). For example, one study showed that nuclear localized HBx stimulates ENHI of HBV (Doria et al. 1995). HBx does not bind to DNA, and HBx activation of RNA polymerase (pol) I–, pol II–, and pol III–dependent promoters has been linked to a direct interaction with some transcription factors as well as stimulation of cellular signal-transduction pathways that regulate transcription (Aufiero and Schneider 1990; Kwee et al. 1992; Wang et al. 1995, 1997, 1998). HBx stimulation of cellular signal-transduction proteins, including Pyk2 and Src kinases, Ras, Raf, and the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK), amino-terminal c-jun kinase (JNK), and p38MAPK, has been linked to HBx activation of various transcription factors (Benn and Schneider 1994; Benn et al. 1996; Andrisani and Barnabas 1999; Klein et al. 1999; Tarn et al. 2001, 2002; Bouchard et al. 2002, 2003; Wang et al. 2008). Transcription factors that are activated by HBx include nuclear factor (NF)-κB, activator protein (AP)1, AP2, C/EBPα, ATF/CREB, SP1, signal transducer and activator of transcription (STAT)3, hypoxia inducible factor (HIF)1α, nuclear factor of activated T cells (NFAT), and E2F (Lucito and Schneider 1992; Lucito 1993; Doria et al. 1995; Williams and Andrisani 1995; Benn et al. 1996; Su and Schneider 1996; Lara-Pezzi et al. 1998; Lee et al. 1998; Tarn et al. 2001, 2002; Waris et al. 2001; Yoo et al. 2003; Wang et al. 2004, 2008). HBx can directly associate with components of the basal transcriptional machinery, including transcription factor (TF)IIB, TFIIH, and TATA-binding protein (TBP) and with transcription factors, such as CREB/ATF, C/EBPα, ATF3, SMAD4, and sterol regulatory element–binding protein (SREBP)1, to increase their activity or affinity for their DNA-binding sites (Williams and Andrisani 1995; Haviv et al. 1996, 1998; Lin et al. 1997a; Choi et al. 1999; Perini et al. 1999; Lee et al. 2001a; Waris et al. 2001; Kim et al. 2007a). It is important to note, however, that many studies of HBx interactions with transcription factors required the use of in vitro protein expression systems or cells in which HBx and a specific transcription factor were overexpressed. Although these types of studies provide insights into HBx activities, confirmation of the interaction of HBx with specific transcription factors in the context of a natural HBV infection is needed to provide definitive proof of the relevance of these interactions.

The ability of HBx to activate cellular transcription pathways has been assessed in various cell lines, including both liver-derived cell lines, such as HepG2, Huh7, and AML12 cells, and non-liver-derived cell lines, such as NIH3T3, HeLa, and Chang cells (reviewed in Bouchard and Schneider 2004; Benhenda et al. 2009; Wei et al. 2010b). Although originally thought to be a liver cell line, careful analysis of Chang cells have confirmed that these cells are likely a HeLa cell contaminant (Nelson-Rees and Flandermeyer 1976). These assays of HBx activities in different cell lines have demonstrated that HBx modulation of transcription promoters and transcription factors can vary in different experimental systems, complicating the evaluation of the precise transcription factors and transcription-signaling pathways that are regulated by HBx during an authentic HBV infection. Moreover, because the assessment of HBx stimulation of transcription has often relied on transient transfections of transcription-reporter plasmids, it remains uncertain whether these studies are directly relevant for HBx effects on endogenous cellular promoters and enhancers (reviewed in Bouchard and Schneider 2004; Benhenda et al. 2009; Wei et al. 2010b). Recent studies have begun to directly assess HBx effects on transcription factor localization to cellular endogenous promoters, and HBx was recently shown to increase the recruitment of CBP/p300 to cellular interleukin (IL)-8 and PCNA promoters (Cougot et al. 2007). Similar types of studies will be essential for confirming that the

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many transcription factors that have been reported to be activated by HBx, and possibly affect expression of endogenous cellular genes, do, in fact, influence transcription from endogenous cellular transcription promoters and enhancers. Although studies that have used cDNA-microarray technologies, chromatin immunoprecipitation (ChIP) methods, or serial analysis of gene expression (SAGE), have clearly shown that HBx can regulate expression of endogenous genes in hepatocytes (Wu et al. 2002; Zhang et al. 2009), a direct correlation of these studies with specific transcription factors activated by HBx in the studies described above awaits further analyses. Importantly, HBx has been shown to stimulate transcription in vivo in hepatocytes. When mice that express a reporter gene controlled by the human immunodeficiency virus (HIV) long terminal repeat (LTR) were crossed with HBx-transgenic mice that express HBx in hepatocytes, transcription of the reporter was increased (Balsano et al. 1994). Moreover, another study reported an increase in HBV core promoter activity by HBx when HBV- and HBx-transgenic mice were crossed (Reifenberg et al. 1999b). Although these results confirm that HBx activates transcription factors in hepatocytes, the mechanisms that underlie this HBx activity and the precise transcription factors that are stimulated in authentic hepatocytes remain incompletely defined.

Recently, HBx has been shown to affect epigenetic-signaling mechanisms (reviewed in Andrisani 2013). HBx can up-regulate expression of DNA methyltransferases DNMT1, DNMT3A1, and DNMT3A2 causing an overall increase in their cellular activity (Qiu et al. 2014). This HBx-induced increase in DNA methyltransferase activity has been linked to decreased expression of E-cadherin and the tumor suppressor p16 (Lee et al. 2005; Jung et al. 2007; Park et al. 2007). Of particular importance to HBV replication, HBx has also been shown to regulate epigenetic signatures on HBV covalently closed circular DNA (cccDNA) minichromosomes (described below), although, in that case, HBx is thought to relieve negative regulation of the cccDNA template, leading to increased transcription (Benhenda et al. 2013). HBx regulation of epigenetic signals provides an additional means for HBx regulation of both cellular and HBV transcription in HBV-infected cells and likely contributes to the many transcriptional effects that have been linked to HBx expression.

HBX AND cccDNA

Soon after infection of a permissive cell, HBV virion DNA, also known as relaxed circular DNA (rcDNA), is converted to cccDNA, which serves as the template for viral transcription (Tuttleman et al. 1986). Two laboratories have reported that conversion of rcDNA to cccDNA occurs in the absence of HBx expression indicating that HBx is not required for this important step in virus replication (Chou et al. 2005; Lucifora et al. 2011). Mutations in the HBV reverse transcriptase do not affect cccDNA formation, and it has been concluded that cellular enzymes are responsible for converting rcDNA into cccDNA (Moraleda et al. 1997; Sohn et al. 2009).

HBV cccDNA is organized as a minichromosome with regularly spaced nucleosomes containing histone and nonhistone proteins (Bock et al. 1994; Newbold et al. 1995). Two populations of cccDNA containing a full or half complement of nucleosomes have been observed, suggesting that cccDNA is dynamic and subject to transcriptional regulation (Newbold et al. 1995). Although cellular chromatin shows a nucleosome repeat of 200 base pairs (bp), HBV cccDNA has 180 bp repeat units (Bock et al. 1994) and duck hepatitis B virus cccDNA a repeat of 150 bp (Newbold et al. 1995). The significance of the more compact viral cccDNA is unknown. cccDNA is present in the nucleus of infected cells at 1 to 50 copies per cell (Newbold et al. 1995; Moraleda et al. 1997; Zoulim 2005; Wang et al. 2011), and is stable throughout the lifespan of the hepatocyte. Antiviral therapy does not eliminate cccDNA, and understanding how the virus regulates expression from the cccDNA template may provide insight into ways to silence cccDNA and prevent reactivation of an inactive or resolved hepatitis B virus infection in which cccDNA is still present.

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The protein composition of cccDNA was determined by antibody reactivity to purified nucleocapsid complexes. These studies established that HBcAg is a regular component of the cccDNA, as are cellular histones H2A, H2B, H3, and H4 (Bock et al. 2001). Other studies used the sensitive ChIP assay to immunoprecipitate (IP) proteins bound to the cccDNA complex, followed by polymerase chain reaction (PCR) detection of cccDNA to which the protein was bound. Using this approach, several positive regulators of gene transcription were shown to be recruited to cccDNA, including histone acetyltransferase CBP, p300, PCAF/GCN5 (Pollicino et al. 2006; Cougot et al. 2007; Belloni et al. 2009), and transcription factor CREB (Cougot et al. 2007). The HBV regulatory HBx protein was also recruited to cccDNA (Belloni et al. 2009), a finding consistent with its proposed role of increasing HBV transcription at the level of cccDNA (Lucifora et al. 2011). Also present on the cccDNA are negative regulators of transcription, histone deacetylase 1 (HDAC1) (Pollicino et al. 2006), hSIRT1 (Belloni et al. 2009), protein phosphatase 1 (PP1) (Cougot et al. 2012), and arginine methyltransferase 1 (PRMTI) (Benhenda et al. 2013).

Gene expression from chromatin is regulated, in part, through histone modification. The amino-terminal tails of the four histones present on the nucleosome surface may be modified by several different enzyme-catalyzed, posttranslational modifications of select amino acids. These modifications include lysine acetylation, lysine and arginine methylation, serine phosphorylation, and attachment of the ubiquitin and small ubiquitin-like modifier (SUMO). Evidence indicates that HBx is associated with epigenetic regulation of cccDNA transcription. The kinetics of HBx binding to the cccDNA complex closely follows that of acetylated H3, a marker of transcriptionally active chromatin and HBV transcription (Belloni et al. 2009). In addition, when cccDNA is formed in the absence of HBx, it is transcriptionally silent (Pollicino et al. 2006; Belloni et al. 2009; Lucifora et al. 2011) and contains nonacetylated histones, histone deactylases HDAC1 and hSirt1 (Pollicino et al. 2006; Belloni et al. 2009), and methylated H4 (Benhenda et al. 2013). When formed in the presence of HBx, the cccDNA is enriched for proteins associated with transcription, such as acetylated H3/H4 and acetyltransferase p300) (Pollicino et al. 2006; Belloni et al. 2009) and hypomethylated H4 (Benhenda et al. 2013). Finally, the addition of HDAC inhibitors to HBV-infected cells blocks the recruitment of HDAC1 to cccDNA, and leads to increased HBV transcription (Pollicino et al. 2006).

The mechanism by which HBx acts on the cccDNA template is complex. In one study, the interaction of HBx with PP1 was associated with a longer half-life of CREB phosphorylation, a positive regulator of transcription (Cougot et al. 2012). Alternatively, the interaction of HBx with the methyltransferase PRMT1 is thought to inhibit methylation of H4 and enhance transcription (Benhenda et al. 2013). Overexpressed PRMT1 inhibited HBV transcription from cccDNA, and this was reversed by the expression of HBx (Benhenda et al. 2013). Because HBx does not bind DNA directly, its interaction with cccDNA must be mediated by another HBV or cellular protein. Interestingly, the cccDNA remains transcriptionally silent in cells that express a mutant HBx protein that is unable to bind cellular DDB1 (Lucifora et al. 2011; van Breugel et al. 2012), suggesting a role for the HBx-DDB1 interaction in the regulation of HBV transcription from the cccDNA template. This is an attractive idea, because DDB1 is a known DNA-binding protein. HBx mutant proteins that do not bind DDB1 are unable to restore HBx-deficient replication in the HBV plasmid replication assay, and the defect occurs at the level of viral transcription (Leupin et al. 2005; Hodgson et al. 2012).

The study of HBx function on a cccDNA template is thought to be more biologically relevant than studies on plasmid DNA. However, the magnitude of the effect of HBx on transcription of the largest HBV mRNA from cccDNA is less than threefold (Belloni et al. 2009), indicating that limitations remain for this model system of HBx function.

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HBx EFFECT(S) ON CELLULAR PATHWAYS

Viruses replicate efficiently in cells, in part by altering the physiology of infected cells to favor viral replication. HBx can stimulate many cellular signal-transduction pathways, and this has been linked to efficient HBV replication (reviewed in Bouchard and Schneider 2004; Benhenda et al. 2009; Wei et al. 2010b).

HBx and Calcium-Signaling Pathways

HBx effects on cellular signal-transduction pathways can involve direct interactions between HBx and cellular signaling proteins and the induction of cellular signal-transduction cascades through modulation of cellular calcium (Ca^{2+}) and reactive oxygen species (ROS) levels (Waris et al. 2001; Bouchard et al. 2002). HBx-induced changes in cytosolic Ca^{2+} and ROS levels have been measured in various liver cell lines (Waris et al. 2001; Chami et al. 2003; Yang and Bouchard 2012). HBx elevation of cytosolic Ca^{2+} activates Pyk2 and Src kinases, Ras, Raf, and mitogen-activated protein kinases (MAPK), STAT3, and various other transcription factors (Bouchard et al. 2002; Tarn et al. 2002). HBx elevation of cellular ROS levels has been linked to activation of NF-κB and STAT3 (Waris et al. 2001). HBx regulation of cytosolic Ca^{2+} stimulates HBV replication in HepG2 cells and cultured primary rat hepatocytes (Bouchard et al. 2002; Gearhart and Bouchard 2010a). HBx regulation of cytosolic Ca^{2+} can be blocked by inhibitors of the mitochondrial permeability transition pore (MPTP) (Bouchard et al. 2002; Gearhart and Bouchard 2010a). The possible role of outer mitochondrial membrane protein voltage-dependent anion channel (VDAC) in functions of the MPTP, and the reported interaction of HBx and VDAC, suggest that HBx regulation of mitochondrial Ca^{2+} signaling may affect its elevation of cytosolic Ca^{2+} levels (Rahmani et al. 2000). This notion was recently supported by studies linking HBx regulation of cytosolic Ca^{2+} levels to store-operated calcium entry (SOCE) mechanisms in HepG2 cells (Yang and Bouchard 2012). In these studies, HBx elevation of cytosolic Ca^{2+} entry required active SOCE channels. Significantly, mitochondria are known to participate in enhanced SOCE channel activity, and HBx elevation of cytosolic Ca^{2+} levels required metabolically active mitochondria (Glitsch et al. 2002; Yang and Bouchard 2012). Interestingly, recent studies have also suggested that mitochondrial antiviral-signaling proteins (MAVS) could be an additional target of HBx (Wei et al. 2010a), and it is possible that mitochondria serve as cellular hubs for some HBx activities.

HBx and Apoptosis

Several studies have shown an effect of HBx on cellular apoptosis. HBx can induce apoptosis, sensitize cells to proapoptotic stimuli or prevent apoptosis, depending on the experimental system used to analyze these HBx effects (reviewed in Rawat et al. 2012). Some studies have shown enhanced apoptosis in hepatocytes in mice expressing HBx, whereas others have not. HBx can block cell death mediated by tumor necrosis factor (TNF)-α, Fas, p53, or transforming growth factor (TGF)-β in some cell lines while promoting apoptosis in other cell lines (Elmore et al. 1997; Kim et al. 1998; Bergametti et al. 1999; Shih et al. 2000; Diao et al. 2001; Lee et al. 2001b; Pan et al. 2001; Shirakata and Koike 2003). In one study, the proapoptotic activity of HBx was through HBx inactivation of c-FLIP, a protein that protects against TNF-α and Fas-mediated apoptosis. However, overexpression of cFLIP did not fully block apoptosis stimulated by HBx, and it is unlikely that cFLIP is the only means through which HBx modulates apoptosis (Kim and Seong 2003). The recognition that HBx interacts with VDAC suggests a possible mechanism of HBx-induced modulation of apoptotic pathways, although this association could be either pro- or antiapoptotic (Rahmani et al. 2000). HBx can induce mitochondrial aggregation and cytochrome *c* release, which could be linked to induction of apoptosis (Takada et al. 1999; Kim et al. 2007b). HBx is antiapoptotic in cultured primary rat hepatocytes; this effect was linked to HBx activation of NF-κB (Clippinger et al. 2009). HBx

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became proapoptotic in cultured primary rat hepatocytes when the activity of NF-κB was blocked, suggesting that HBx can be either pro- or antiapoptotic depending on the status of NF-κB.

HBx and the Cell Cycle

Many viruses encode proteins that can stimulate the cell cycle, and the need for this function for HBV is suggested by the fact that HBV infects nondividing hepatocytes. Indeed, HBx can regulate cell proliferation, although the precise impact of HBx has varied in different experimental systems. HBx can cause cells to enter the cell cycle and stall at the G_1/S border or progress more rapidly through the cell cycle, depending on the experimental system used to analyze this HBx activity (reviewed in Madden and Slagle 2001; Casciano et al. 2012). HBx had different effects in differentiated and dedifferentiated hepatocytic cells that were derived from the same parental cell line. The differentiated cells displayed HBx-dependent G_1, S, and G_2/M progression; the dedifferentiated cells displayed HBx-dependent G_1 and S phase entry but paused in S phase (Lee et al. 2002). HBx caused cultured primary rat hepatocytes to exit G_0 but stall in G_1; this HBx effect was directly linked to HBx stimulation of HBV replication in these cells (Gearhart and Bouchard 2010a,b). In cultured primary rat hepatocytes, HBx decreased expression of the cell-cycle inhibitor proteins, p15 and p16, which block entrance into G_1, but elevated levels of the cell-cycle inhibitor proteins, p21 and p27, which have an activating role in G_1 but inhibit progression into S phase. HBx also increased the level of cyclin D1 and the activity of cyclin-dependent kinase 4 (CDK4), proteins important in G_1 progression, but did not affect levels of the S phase proteins and inhibited CDK2 activity, which is required for progression beyond G_1 (Gearhart and Bouchard 2010a,b). Overall, these studies showed that, for efficient HBV replication, hepatocytes must exit G_0 and enter G_1, but then remain in the G_1 phase of the cell cycle. Similar HBx effects on cell proliferation pathways were observed in human hepatocytes (Gearhart and Bouchard 2011). Three groups used HBx transgenic mice to analyze HBx effects on hepatocyte regeneration; two groups showed that HBx blocked liver regeneration (Tralhao et al. 2002; Wu et al. 2006), whereas one group showed that HBx caused cell-cycle entry of a subpopulation of hepatocytes (Hodgson et al. 2008).

HBx and DDB1

HBx can also regulate responses to and repair of damaged DNA. One HBx-binding partner, the damaged DNA-binding protein 1 (DDB1) (Lee et al. 1995; Sitterlin et al. 1997) is an adaptor protein for the Cullin 4-DDB1 E3 ligase. DDB1 functions, in part, through its interactions with DDB1 cullin adaptor factors (DCAFs) that bind to a specific location on the DDB1 molecule. DCAF proteins recruit substrates for E3-mediated ubiquitination and degradation (reviewed in McCall et al. 2005; Lee and Zhou 2007; O'Connell and Harper 2007). Because HBx shares a 16-amino acid motif with these DCAFs (Keasler and Slagle 2008; Li et al. 2010), it is possible that HBx may displace one or more DCAFs from the Cul4/DDB1 complex, thereby altering the spectrum of downstream cellular proteins targeted for degradation. Studies from two laboratories reported that HBx can displace at least two different DCAFs from DDB1 in transfected cells in culture (Bergametti et al. 2002; Li et al. 2010), although the downstream targets affected by this displacement are not known.

There is evidence that the binding of HBx (or WHx) to DDB1 is important in virus replication. WHV is highly homologous to HBV and encodes an X protein (WHx) that is similar to HBx and required for WHV replication. Viremia was not observed when woodchucks were infected with the WHV engineered to encode WHx that could not bind to DDB1 (Sitterlin et al. 1997). In woodchucks infected with a WHV mutant that encoded WHx, which could not bind DDB1, the few woodchucks that developed viremia were found to have reverted to wild-type WHx, suggesting that the authentic WHx–DDB1 interaction was essential for virus

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replication in vivo. A similar conclusion was reached in studies using the HBV plasmid replication assay in HepG2 cells; HBx-deficient HBV replication could be restored by wild-type HBx, but not by HBx mutants that were unable to bind DDB1 (Leupin et al. 2005; Hodgson et al. 2012). The HBx–DDB1 interaction may be important for HBV transcription (described above) or for the cellular response to damaged DNA (Becker et al. 1998), but other possible functions should be considered. Several viruses encode proteins that bind to DDB1 as part of their strategy for virus replication (reviewed in Barry and Früh 2006; Dehart and Planelles 2008). The *Paramyxovirus* Simian Virus 5 (SV5) regulatory V protein binds to DDB1 (Lin et al. 1998) and redirects the E3 ligase to ubiquitinate and degrade STAT1, thereby inactivating host interferon signaling (Precious et al. 2005). The murine herpesvirus 68 interacts with the Cul4A-DDB1^{COP9} complex to activate the DNA damage signal (Liang et al. 2006). The HIV-1 regulatory Vpr protein binds DDB1 indirectly (through Cul4A-DDB1^{DCAF1}), and redirects DDB1 to induce G_2 cell-cycle arrest (Schrofelbauer et al. 2007). The DDB1 function targeted by HBx is an important unanswered question.

HBx and Cancer

Certain properties of HBx, such as its ability to regulate cellular signal-transduction pathways, affect cell-cycle progression, and activate damaged DNA responses, raise the possibility that HBx may contribute to the development of HBV-associated hepatocellular carcinoma (HCC). A chronic HBV infection remains the most common cause of HCC (Mittal and El-Serag 2013), and the inflammatory response to HBV infection and viral integration are clearly central to any model of HBV-associated HCC. However, there is also evidence that HBx can contribute to the carcinogenesis process (reviewed in Riviere et al. 2014). Studies in HBx transgenic mice have yielded complex results. Some lineages of HBx mice develop HCC (Kim et al. 1991; Yu et al. 1999; Wu et al. 2006), whereas other lineages do not (Lee et al. 1990; Billet et al. 1995; Reifenberg et al. 1999a; Klein et al. 2003). A close examination of the studies showing HCC revealed complications of spontaneous HCC in nontransgenic control mice (Kim et al. 1991), the presence of steatosis (Wu et al. 2006), or a possible misdiagnosis of biliary cysts rather than HCC (Dirsch et al. 2004). Despite the paucity of data on HBx as an oncogene in vivo, there is agreement that HBx can cooperate with various HCC risk factors to further increase the incidence of HCC (Dandri et al. 1996; Slagle et al. 1996; Klein et al. 2003; Zhu et al. 2004; Keasler et al. 2006; Wang et al. 2012). Precisely how HBx effects HCC development in the context of a HBV replication awaits the development of model systems in which this process can be studied during a natural HBV infection and remains an area of active and important investigation.

The Multifunctional Nature of HBx

Considering the small size of its genome, HBV must rely heavily on the host cell to accomplish its life cycle (Fig. 2). HBx is the only regulatory protein encoded in the HBV genome and participates in generating a cellular environment that is conducive to HBV replication; thus, it is not surprising that HBx has been assigned numerous functions (reviewed in Benhenda et al. 2009; Wei et al. 2010b; Lucifora and Protzer 2012). HBx has been implicated in viral and cellular transcription, DNA repair, cellular proliferation, autophagy/mitophagy, and apoptosis (Tian et al. 2011, and as described above). The ability of HBx to reside in different subcellular compartments is one important feature of its multifunctional role during HBV replication. Cytoplasmic HBx transactivates cellular-signaling pathways (e.g., MAPK, JNK, p38, JAK-STAT, pathways, etc.) (Klein and Schneider 1997; Tarn et al. 2002), and nuclear HBx was reported to interact with components of the basal transcription machinery (RPB5, TFIIB, TBP) (Cheong et al. 1995; Qadri et al. 1995; Haviv et al. 1996) and specific transcription factors to regulate host genes (Lucito and Schneider 1992; Lucito 1993). In addition, the ability of HBx to interact with many different cellular partners could

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mediate its pleiotropic effects (reviewed in Ganem and Schneider 2001; Seeger et al. 2013). A comprehensive description of all cellular proteins that interact with HBx and all cellular-signaling pathways that are modulated by HBx is beyond the scope of this review; the reader is referred to reviews that have extensively described HBx interaction partners and HBx functions (Benhenda et al. 2009; Wei et al. 2010b).

Although the many functions ascribed to HBx have raised the question of how one protein could have so many effects, it is important to note that a broad array of activities in viral regulatory proteins is neither unexpected nor unprecedented. Some confusion regarding HBx effects has probably been caused by the myriad of experimental systems and HBx expression methods that have been used to study HBx functions. For example, HBx activities have been analyzed with HBx that is expressed alone and in the context of HBV replication and in cell lines of liver and nonliver origin, in cultured primary human, mouse, and rat hepatocytes, and in hepatocytes in the livers of normal mice and mice with humanized livers (reviewed in Bouchard and Schneider 2004; Benhenda et al. 2009; Wei et al. 2010b). Because these studies have often analyzed the end point of complex cellular signal-transduction pathways, observed HBx effects likely represent HBx activities that are specific to a particular cellular environment. The results of these many studies, and recent attempts to identify fundamental HBx activities, suggest the pleiotropic effects of HBx in various experimental systems may be controlled by a few fundamental HBx activities that can ultimately have different effects in different experimental systems. A key remaining area of investigation is to identify HBx activities in the context of HBV replication in biologically relevant in vivo and ex vivo primary hepatocyte systems. Although overexpression of HBx has also been considered as a complicating factor that could affect HBx activities observed in some studies, the precise level of HBx expression during HBV replication is unknown. Recent studies that have analyzed the in vivo activity of HBV promoters and enhancers suggest that the levels of HBx in an authentic infection may have been underestimated (Du et al. 2008). HBx expression has been reported as being low during HBV replication, but these conclusions were based on comparison to expression of other HBV proteins with antibodies of unknown affinity for their targets (reviewed in Bouchard and Schneider 2004; Benhenda et al. 2009; Wei et al. 2010b). Because the affinity of available HBx antibodies has not been determined, analysis of HBx protein levels can only be used to provide an estimate of HBx expression; moreover, comparison to expression level of other HBV proteins is inaccurate in the absence of affinity calculations for the antibodies used to detect these proteins. It, therefore, remains unknown whether HBx "overexpression" should be considered to have affected reported HBx activities, and recent studies that have compared HBx expressed alone (overexpressed) or in the context of HBV replication reported identical HBx effects in either context (Clippinger and Bouchard 2008; Clippinger et al. 2009; Gearhart and Bouchard 2010a; Yang and Bouchard 2012; Kim et al. 2013).

Finally, predictive models of HBx structure suggest that its amino-terminal region is unstructured, and it is possible that this may contribute to the ability of HBx to interact with a large number of cellular proteins and affect numerous cellular signal-transduction pathways (Hernandez et al. 2012). Interestingly, a number of recent reports that have addressed the multifunctional nature of viral regulatory proteins have shown that some multifunctional viral proteins can undergo structural rearrangements, particularly in regions of the protein characterized as unstructured, to interact with various cellular proteins and affect different aspects of the viral life cycle (Bornholdt et al. 2013; Ferreon et al. 2013). Moreover, some viral proteins can assemble and function as protein polymers that have multiple functions in their virus life cycle (Ou et al. 2012). Consequently, it is possible that the multiple functions ascribed to HBx may derive from structural rearrangements of HBx, possibly in its unstructured amino-terminal region, which could occur throughout the HBV life cycle.

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CONCLUDING REMARKS

HBx can interact with multiple cellular proteins to either directly or indirectly regulate cellular and HBV gene expression, and can modulate numerous cellular signal-transduction pathways. Most of what we know about regulation of HBV transcription by HBx and of HBx modulation of cellular-signaling factors has been learned from transfected cells in culture, a milieu that differs dramatically from the in vivo setting. HBV is highly adapted for replication in well-differentiated hepatocytes with abundant liver-enriched transcription factors, and studies in established liver cell lines are unlikely to completely mimic the cellular environment encountered by HBV during infection of hepatocytes. Consequently, a precise understanding of the role of HBx during HBV replication and the myriad of HBx effects on hepatocyte physiology awaits the development of experimentally tractable in vivo model systems for analyzing HBx activities during a natural HBV infection. Interestingly, many of the HBx activities important for stimulating HBV replication may inadvertently contribute to the development of HBV-associated HCC. Defining the mechanisms that regulate HBV replication is essential for identifying novel therapeutic targets for treating individuals with a chronic HBV infection and at high risk for developing HCC.

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