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Modulation of Cell Proliferation Pathways by the Hepatitis B Virus X Protein: A Potential Contributor to the Development of Hepatocellular Carcinoma

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1. Introduction

Globally, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third highest cause of cancer-associated deaths. Although the development of HCC has been linked to exposure to various toxins or infectious agents, the majority of HCC cases are associated with chronic hepatitis B virus (HBV) infections [reviewed in (Block et al, 2003; Seeger et al, 2007)]. Worldwide, there are an estimated 350 million cases of chronic HBV infections, and approximately 25% of chronically HBV-infected individuals will eventually develop HCC [(Beasley et al, 1981); reviewed in (Seeger et al, 2007)]. The high global incidence of chronic HBV infections, high mortality rate of individuals with HCC, increased prevalence of HCC, and the close association between chronic HBV infections and HCC development have generated intense interest in understanding the molecular mechanisms that underlie the development of HBV-induced HCC. In this chapter, we provide a review of HBV biology and potential mechanisms that link a chronic HBV infection to the development of HCC. We specifically focus on activities of the HBV X protein (HBx), a multifunctional HBV protein that can alter hepatocyte physiology and stimulate HBV replication. While a brief survey of HBx activities that could influence HCC development is provided, we emphasize HBx regulation of intracellular calcium signaling and cell proliferation pathways as HBx activities that could potentially influence hepatocyte transformation.

2. Hepatitis B virus

HBV is a member of the *Hepadnaviridae,* a family of hepatotropic viruses that predominately infect hepatocytes in their respective hosts; similar viruses have been isolated from apes, woodchucks, squirrels, ducks, geese, and cranes [reviewed in (Seeger et al, 2007)]. HBV has a highly compact DNA genome of about 3200 nucleotides in length that contains four overlapping open reading frames (ORFs); every nucleotide of the genome is in at least one

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open reading frame (Figure 1). The four overlapping ORFs of the HBV genome encode seven proteins: the reverse transcriptase/polymerase, the pre-core (E antigen) and core (capsid) proteins, three envelope (large, middle, and small hepatitis B surface antigens) proteins, and the nonstructural X protein (HBx) [reviewed in (Seeger et al, 2007)]. The cell-surface receptor of HBV that facilitates HBV infection of hepatocytes has not been conclusively identified, and the mechanism by which HBV enters hepatocytes is not clear. Upon infection of hepatocytes, the encapsidated, partially double-stranded DNA genome is transported to the nucleus, where it is converted into a covalently closed, double-stranded, circular DNA (cccDNA). cccDNA is the template for all HBV RNA transcripts; the transcripts are exported out of the nucleus into the cytoplasm and are translated to form the HBV pre-core, core, envelope, reverse transcriptase/polymerase, and HBx proteins. The largest HBV RNA transcript, the pregenomic RNA (pgRNA), is packaged with the reverse transcriptase into viral capsids in the cytosol and is reverse transcribed to generate the partially double-stranded HBV DNA genome [reviewed in (Seeger et al, 2007)]. HBV viral capsids containing the replicating genome bud into the endoplasmic reticulum by envelopment within the HBV envelope proteins and are secreted from the infected hepatocyte [reviewed in (Nguyen et al, 2008; Seeger et al, 2007)].

2.1 Model systems for studying HBV infections

Each member of the hepadnavirus family has a narrow host range that is thought to be defined primarily by the interaction between the virus and a specific receptor that is present on the surface of host hepatocytes [reviewed in (Seeger et al, 2007)]. Available cell culture systems for studying the life cycle of the *Hepadnaviridae* are limited. Typically, members of the hepadnavirus family can only directly infect hepatocytes within the liver of their respective avian or mammalian hosts or cultured, well-differentiated primary hepatocytes that are derived from these hosts; this has hampered the capabilities of researchers to study a natural HBV infection [reviewed in (Koike, 2009; Seeger et al, 2007)]. Chimpanzees are the most relevant animal model for studying the consequences of an HBV infection; however, due to cost and ethical reasons, studies in chimpanzees are limited (Prince & Brotman, 2001). HBV infections in chimpanzees also do not completely mimic all aspects of an HBV infection in humans; chimpanzees chronically infected with HBV usually do not develop liver cirrhosis or HCC (Prince & Brotman, 2001). HBV-transgenic mice have served as an important small animal model for studying *in vivo* HBV replication and immune-mediated HBV clearance from hepatocytes [(Guidotti et al, 1995); reviewed in (Guidotti & Chisari, 2006)]. However, because HBV-transgenic mice contain a copy of the HBV genome that is integrated into the genome of all hepatocytes in these mice, the consequences of chronic inflammation for the development of HBV-associated HCC cannot be studied in this model [reviewed in (Bouchard & Navas-Martin, 2011)]. More recently, human hepatocyte chimeric mice, which were generated by the replacement of the majority of the mouse hepatocytes with implanted human hepatocytes, have been used to study HBV infections in what may become a more experimentally tractable and relevant model than other currently available small animal model systems (Tsuge et al, 2005). Mice with humanized-livers that were inoculated with HBV had high levels of HBV viremia that lasted for up to 22 weeks (Tsuge et al, 2005). Hydrodynamic transfection of the HBV genome into mouse livers has also been used as a method for studying HBV replication in hepatocytes; however, due to rapid clearance of the virus, persistent HBV infection cannot be studied in this system (Keasler et al, 2007). The paucity of *in vivo* models for studying direct HBV infections, and

the limited availability of cultured primary human hepatocytes, has lead many researchers to study HBV replication and the activities of HBV-encoded proteins in immortalized or transformed liver cell lines and in cultured primary hepatocytes derived from small animal models such as rats or mice [reviewed in (Bouchard & Navas-Martin, 2011; Seeger et al, 2007)]. Use of these systems necessitates the bypass of the initial receptor-mediated infection of the cell by direct transfection of the HBV DNA genome. Although studies in immortalized or transformed cells have served as powerful models for studying various aspects of HBV replication and the functions of HBV-encoded proteins, these studies have also demonstrated that the activities of HBV proteins may vary in different cellular contexts [reviewed in (Bouchard & Navas-Martin, 2011; Neuveut et al, 2010)]. Studies in cultured primary hepatocytes have begun to clarify HBV replication strategies and the function of HBV proteins in a more relevant context [reviewed in (Bouchard & Navas-Martin, 2011)]. Recently, cultured primary rat hepatocytes have been used to study HBV replication and functions of the HBx protein (Clippinger & Bouchard, 2008; Clippinger et al, 2009; Gearhart & Bouchard, 2010a; Gearhart & Bouchard, 2010b); HBx activities in cultured primary rat hepatocytes were similar to HBx activities in cultured primary human hepatocytes, supporting the use of cultured primary rat hepatocytes as a good model system for studying the impact of HBV on hepatocyte physiology (Gearhart & Bouchard, 2011).

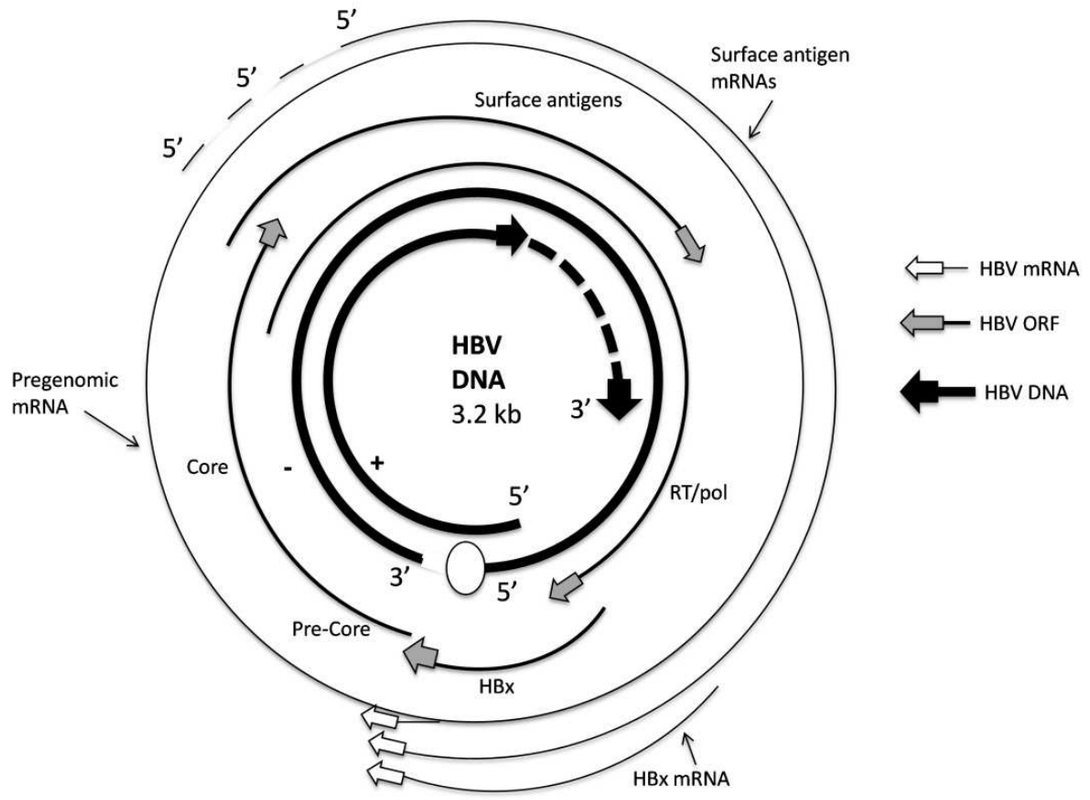

Fig. 1. Schematic depiction of the HBV genome. HBV DNA is denoted by bold lines with black arrows, HBV open reading frames (ORFs) are represented by inner arrows shown in gray, and HBV mRNAs are represented by outer arrows shown in white. See text for detailed description.

2.2 HBV and hepatocellular carcinoma

Common modes of HBV transmission include exposure to HBV-contaminated blood, unsafe injection practices, sexual contact with an HBV-infected individual, and perinatal transmission from an HBV-infected mother to her child (WHO, 2008). HBV infections are classified as acute, chronic, or occult. In most people with acute or short-term HBV infections, the infection is resolved by immune-mediated viral clearance. The inflammatory response to the HBV infection causes many of the symptoms that have been associated with hepatitis; these symptoms include jaundice, fatigue, nausea, vomiting, and abdominal pain [(WHO, 2008); reviewed in (Seeger et al, 2007)]. Chronic HBV infections are characterized by the continued presence of detectable levels of the HBV small envelope protein in the blood of an HBV-infected individual for more than six months [reviewed in (Lok & McMahon, 2001)]. Chronic infections with either HBV or the hepatitis C virus (HCV) are estimated to account for more than 80% of primary liver cancers; approximately 60% of these liver cancers are attributed to chronic HBV infections and 40% to chronic HCV infections [reviewed in (El-Serag & Rudolph, 2007; Koike, 2009)]. Currently, the geographic locations with the highest incidence of HCC are areas where HBV infection is highly endemic [reviewed in (Pang et al, 2006)]. There are eight known genotypes of HBV, and mounting evidence suggests that increased persistence of an HBV infection and a greater risk for HCC development may be influenced by the HBV genotype in the infected individual [reviewed in (Chemin & Zoulim, 2009)]. Environmental factors such as alcohol consumption, aflatoxin exposure, and tobacco use increase the risk for developing HCC in HBV-infected individuals [reviewed in (Chemin & Zoulim, 2009)].

Although the association between chronic HBV infections and HCC development is clear, the mechanisms that link a chronic HBV infection to HCC development are incompletely understood. Three potential mechanisms have been commonly invoked as consequences of an HBV infection that could contribute to HCC development. The first mechanism that may contribute to the development of HBV-associated HCC is persistent liver inflammation and hepatocyte proliferation that is caused by recurrent immune-mediated destruction of HBV-infected hepatocytes and concomitant liver regeneration in chronically HBV-infected individuals [reviewed in (Bouchard & Navas-Martin, 2011; Guidotti & Chisari, 2006)]. Persistent liver inflammation can cause fibrosis and cirrhosis and may eventually select for hepatocytes that have accumulated tumorigenic properties [reviewed in (Bouchard & Navas-Martin, 2011; Chemin & Zoulim, 2009)]. Chronic liver inflammation, the associated elevation of reactive oxygen species (ROS), and the potential for ROS to cause DNA damage may also produce a more cancer-prone environment [reviewed in (Chemin & Zoulim, 2009)]. The second mechanism that has been proposed to contribute to the development of HBV-associated HCC is the possible consequence of HBV genome integration into the host genome, which could cause genetic or epigenetic changes and genomic instability [reviewed in (Chemin & Zoulim, 2009; Neuveut et al, 2010)]. Integration of the HBV genome into the host genome could potentially result in the loss of tumor suppressive functions and/or the gain of tumor-promoting activities [reviewed in (Chemin & Zoulim, 2009)]. The third mechanism that has been proposed to be involved in the development of HBV-associated HCC, and the primary focus of this chapter, is the alteration of hepatocyte physiology and stimulation of HBV replication that is linked to expression of the HBV HBx protein [reviewed in (Bouchard & Navas-Martin, 2011; Koike, 2009; Neuveut et al, 2010)]. The multifunctional HBx protein has been shown to affect numerous cellular signaling pathways

that could influence HBV replication, hepatocyte transformation, and HCC development [reviewed in (Benhenda et al, 2009; Koike, 2009; Neuveut et al, 2010)]. Although this chapter focuses on HBx activities, it is important to note that in the context of an HBV infection, multiple mechanisms are likely to influence HCC development.

3. HBx

HBx is a 154 amino acid, 17 kDa, multifunctional protein that is encoded by the smallest open reading frame of the HBV genome. HBx can stimulate HBV replication and modulate intracellular calcium signaling, cell proliferation and apoptotic pathways, signal transduction pathways, and the activity of various transcription factors and the proteasome (Figure 2) [reviewed in (Benhenda et al, 2009; Bouchard & Schneider, 2004; Koike, 2009)]. Because HBx activities appear to be influenced by the cell type and the method of HBx expression used in a study, not all HBx functions in normal hepatocytes and in the context of HBV replication are completely understood. Most studies that have assessed the effects of HBx have been performed in immortalized or transformed cells, when HBx was expressed at higher levels than observed during HBV replication, and when HBx was expressed outside of the context of HBV replication. Because HBx is expressed at low levels during HBV replication, it has been difficult to establish systems in which HBx interacting partners or HBx activities can be easily studied in the context of HBV replication. Consequently, the use of various cellular model systems to study HBx has sometimes identified seemingly discrepant HBx activities. It is important to note however, that many studies that have analyzed HBx activities in systems where HBx is expressed in the context of HBV replication and in normal hepatocytes have confirmed HBx activities that were identified when HBx

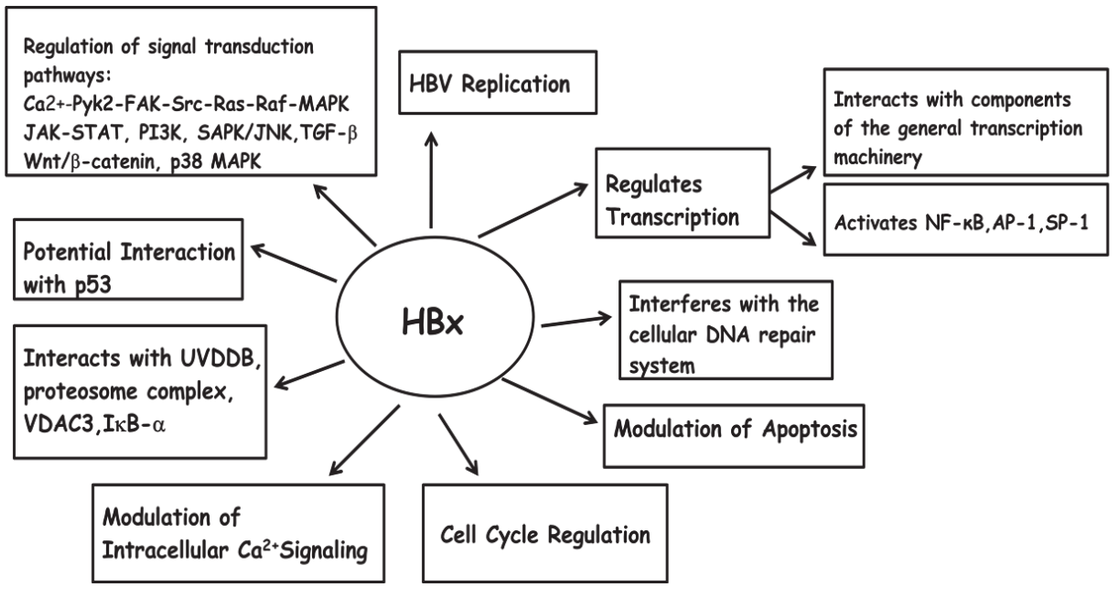

Fig. 2. Schematic representation of HBx activities. HBx is a multifunctional protein known to regulate HBV replication, numerous signal transduction pathways, the cell cycle, apoptosis, and transcription. HBx can also bind multiple cellular proteins. One or more of these HBx activities could contribute to the development of HBV-associated HCC. See text for references and details.

was overexpressed out of the context of HBV replication, suggesting that studies in various cellular systems can provide valuable insights into HBx activities [reviewed in (Benhenda et al, 2009; Bouchard & Schneider, 2004; Koike, 2009)]. Moreover, because HBx is often expressed in HBV-associated liver tumor cells even when expression of other HBV proteins is not detectable (Wang et al, 2004b; Wollersheim et al, 1988), studies in immortalized or transformed cells may help identify HBx activities that could be present in transformed cells but absent in normal hepatocytes. Overall, it is likely that studies in immortalized or transformed cells, in cultured primary hepatocytes, in the livers of available animal models, and when HBx is expressed in the absence of other HBV proteins and in the context of HBV replication each contribute important information regarding the various activities of this protein. Considering the compact nature of the HBV genome, the limited number of proteins encoded by the HBV genome, and the necessity for these proteins to perform their functions in an environment that may not favor viral replication, it is not surprising that HBV proteins such as HBx, which is the only regulatory protein encoded by the HBV genome, have multiple functions that impact HBV replication and cellular physiology.

3.1 HBx and HBV replication

Many studies have indicated that replication of mammalian hepadnaviruses is stimulated by their respective X proteins. Two different studies performed in woodchucks showed that the woodchuck hepatitis virus (WHV) X protein (WHx) is absolutely required for WHV replication in the livers of woodchucks (Chen et al, 1993; Zoulim et al, 1994); however, in one dissenting study, another group detected a very low level of WHV replication in woodchucks infected with an WHx-deficient WHV mutant (Zhang et al, 2001). Evidence that HBx stimulates HBV replication has been generated from studies in various mouse models and cell culture systems. Transgenic mice were generated with either a wild-type HBV genome or a mutant form of the HBV genome that did not express HBx; HBV replication was detected in both the wildtype HBV and mutant HBV-transgenic mice (Xu et al, 2002). However, when the HBx-deficient HBV-transgenic mice were bred with HBx-transgenic mice, higher levels of circulating HBV viremia and higher levels of HBV core and envelope proteins were detected in the double transgenic mice than in their HBx-deficient, HBV-transgenic counterparts (Xu et al, 2002). These studies suggest that although HBx may not be absolutely required for HBV replication in this model system, HBx does enhance HBV replication in the livers of these mice. An important caveat to the studies in HBV-transgenic mice is that these mice do not produce nuclear HBV cccDNA and may not recapitulate all aspects of authentic HBV replication (Guidotti et al, 1995). Additional evidence that HBx stimulates HBV replication has come from a study where mice were hydrodynamically injected with a plasmid encoding the wild-type HBV genome or a mutant HBx-deficient HBV genome (Keasler et al, 2007). The results of these studies demonstrated that although HBx was not absolutely required for HBV replication, there was a very significant decrease in HBV replication in mice injected with the HBx-deficient HBV as compared to mice injected with the wildtype HBV genome (Keasler et al, 2007). Interestingly, when a plasmid encoding HBx was co-injected with a plasmid encoding the mutant HBx-deficient HBV genome, HBV replication levels were restored to those seen in mice injected with the plasmid encoding wild-type HBV (Keasler et al, 2007). Finally, studies in mice with humanized livers showed that after direct infection with wild-type HBV or an HBx-deficient HBV, HBV replication was only seen in the livers of mice that were infected

with wild-type HBV (Tsuge et al, 2010; Tsuge et al, 2005). HBx expression also stimulates HBV replication in HepG2 cells, a human hepatoblastoma cell line, and in cultured primary rat hepatocytes [reviewed in (Bouchard & Schneider, 2004)]. Although most studies indicate that HBV replication in Huh7 cells, a human hepatoma cell line, is not regulated by HBx expression, a recent study demonstrated that HBx can also stimulate HBV replication in Huh7 cells (Lim et al, 2010). Many of the signaling pathways that are modulated by HBx, and will be discussed in the following sections of this chapter, have also been shown to influence HBV replication. HBx modulation of intracellular calcium signaling, activation of the Proline-rich tyrosine kinase 2 (Pyk2)/Focal adhesion kinase (FAK)-Src-Ras-Raf-MAPK signaling pathway, regulation of transcription pathways, interaction with ultraviolet DNA damage binding protein 1 (UVDDB1), and association with proteasome factors affect HBx regulation of HBV replication (Bouchard et al, 2001b; Gearhart & Bouchard, 2010b; Klein et al, 1999; Leupin et al, 2005; Tan et al, 2009; Tang et al, 2005; Zhang et al, 2004). Overall, studies in various model systems suggest that the X proteins of mammalian hepadnaviruses have an important stimulatory role during replication of mammalian hepadnaviruses.

3.2 HBx and hepatocellular carcinoma

HBx expression and activities are thought to be major contributing factors in the development of HBV-associated HCC; however, the exact contribution of HBx to HCC development is unknown. One important clue that implicates HBx in the development of HBV-associated HCC is that chronic infections of birds with avian hepadnaviruses are not associated with the development of HCC [reviewed in (Seeger et al, 2007)]. The avian hepadnaviruses either do not encode an X protein or encode an X protein that is highly divergent from the X proteins of mammalian hepadnaviruses (Mandart et al, 1984; Sprengel et al, 1988). The mammalian hepadnaviruses all encode an X protein, and only infections with mammalian hepadnaviruses are associated with the development of HCC in their respective hosts [reviewed in (Seeger et al, 2007)]. Additional evidence for the involvement of HBx in HCC development has come from studies in HBx-transgenic mice. Some studies have shown that HBx expression can directly cause HCC in HBx-transgenic mice (Kim et al, 1991; Yu et al, 1999). In contrast, other researchers have found that HBx expression alone is not sufficient for the development of liver tumors in HBx-transgenic mice but can sensitize these mice to chemical- or oncogene-induced HCC (Madden et al, 2001; Slagle et al, 1996; Terradillos et al, 1997; Zhu et al, 2004). While the reason for the observed differences in tumor development in the various HBx-transgenic mice has not been completely explored and may be related to the genetic background of the mice and/or the level of HBx expression, studies in these HBx-transgenic mice strongly support the notion that HBx can have at least a co-factor role in the development of HCC. Importantly, a co-factor role for HBx in the development of HBV-associated HCC is more consistent with the biology of HCC development in chronically HBV-infected individuals; development of HCC in chronically HBV-infected individuals can take decades to arise [reviewed in (Seeger et al, 2007)]. The observation that HBV-associated HCC requires decades to arise suggests that HBV does not encode a strongly oncogenic protein but instead encodes proteins that can cause subtle changes to hepatocyte physiology that could sensitize hepatocytes to other oncogenic signals [reviewed in (Bouchard & Navas-Martin, 2011; Neuveut et al, 2010)]. The activities of HBx that could influence HCC development are discussed below.

3.3 Intracellular localization of HBx

The primary intracellular location of HBx is the cytoplasm, although a small fraction of HBx can be found in the nucleus of cells (Doria et al, 1995; Haviv et al, 1998b; Henkler et al, 2001; Siddiqui et al, 1987; Urban et al, 1997; Vitvitski-Trepo et al, 1990). The localization of HBx is dependent on its level of expression. When HBx is expressed at very low levels, it is mainly localized to the nucleus, and when HBx is expressed at high levels, its localization is mainly cytoplasmic (Henkler et al, 2001). Many studies have also shown that a fraction of cytosolic HBx localizes to the mitochondria and can interact with the voltage-dependent anion channel (VDAC) 3, a component of the mitochondrial permeability transition pore (MPTP) (Clippinger & Bouchard, 2008; Henkler et al, 2001; Huh & Siddiqui, 2002; Kim et al, 2007; Rahmani et al, 2000; Shirakata & Koike, 2003; Takada et al, 1999). Cytoplasmic, nuclear, and mitochondrial HBx localization have been directly linked to HBx activities that regulate specific cellular signal transduction or transcription pathways [reviewed in (Ma et al, 2011)].

3.4 HBx interacts with multiple cellular proteins

HBx has been reported to interact with various cellular proteins, such as components of the proteasome complex (Fischer et al, 1995; Huang et al, 1996), UVDDB1 and 2 (Becker et al, 1998; Lee et al, 1995; Sitterlin et al, 2000)), the cell cycle regulatory protein p53 (Elmore et al, 1997; Feitelson et al, 1993; Wang et al, 1994), the NF-κB inhibitory protein IκB-α (Weil et al, 1999), and VDAC3 (Rahmani et al, 2000; Rahmani et al, 1998), to name a few. HBx can also interact with components of the general transcription machinery such as the TATA-binding protein (TBP), the RBP5 subunit of RNA polymerase, and the general transcription factors TFIIB and TFIIH [(Aufiero & Schneider, 1990; Barnabas et al, 1997; Cheong et al, 1995; Haviv et al, 1998a; Haviv et al, 1998b; Maguire et al, 1991; Williams & Andrisani, 1995; Yang et al, 1999); reviewed in (Bouchard & Schneider, 2004)]. While the potential association of HBx with many cellular proteins could explain the multifunctional nature of HBx, because many studies that have analyzed HBx-interacting proteins were conducted in systems in which both HBx and these proteins were overexpressed, whether all of these interactions exist in normal hepatocytes when HBx is expressed during HBV replication remains to be determined. Due to space limitations, the following sections will focus on the interaction of HBx with UVDDB1 and 2 and p53; these interactions have been studied by various groups and may play an important role in HBV-induced HCC (Feitelson et al, 1993; Lee et al, 1995; Sitterlin et al, 2000; Wang et al, 1994). A more extensive discussion of proteins that may interact with HBx can be found in recently published reviews of this topic (Arbuthnot et al, 2000; Benhenda et al, 2009; Wei et al, 2010; Zhang et al, 2006).

3.4.1 HBx and UVDDB1 and 2

UVDDB1 associates with UVDDB2 to form the UV-DDB complex, which functions in nucleotide excision repair. The UVDDB complex also interacts with the transcription factor E2F1, indicating a role for the UVDDB complex in both DNA repair and cell cycle control [(Datta et al, 2001; Hayes et al, 1998); reviewed in (Butel et al, 1996)]. Results from yeast-2-hybrid screens indicated that HBx can directly interact with UVDDB1 (Lee et al, 1995; Lin-Marq et al, 2001; Sitterlin et al, 2000). Further evidence for an interaction between hepadnavirus X proteins and the UVDDB complex was provided from studies performed with WHV; these studies confirmed an interaction between WHx and UVDDB1 (Bergametti

et al, 2002). Disruption of the WHx-UVDDB1 interaction inhibited WHV replication in woodchucks, indicating that WHx and UVDDB1 must associate in order for WHV replication to occur *in vivo* (Sitterlin et al, 2000). WHx was also shown to interact with UVDDB2. The interaction between WHx and UVDDB1 stabilized WHx by protecting it from proteasomal degradation, but the interaction between UVDDB1 and UVDDB2 and between HBx and UVDDB2 counteracted this protection (Bergametti et al, 2002). In one study, HBx altered UVDDB1 activity, resulting in decreased cell viability (Lin-Marq et al, 2001). Similar to the required interaction of WHx and UVDDB1 for WHV replication, HBx association with UVDDB1 was also shown to be required for HBV replication (Sitterlin et al, 2000). HBx expression diminished the innate ability of cells to repair DNA damage, and the results of a study in HepG2 cells suggested that the binding of HBx to UVDDB1 inhibits normal UVDDB1 functions and can result in chromosome segregation defects (Becker et al, 1998; Martin-Lluesma et al, 2008). Results from studies in HBx-transgenic mice, however, indicated that the expression of HBx did not significantly increase the accumulation of spontaneous mutations, implying that although the interaction between HBx and UVDDB1 may inhibit the ability of cells to repair DNA damage, this interaction is not directly mutagenic (Madden et al, 2000). Interestingly, it has been suggested that the interaction between HBx and UVDDB1 may be involved in the HBx-related cell cycle arrest at the G1/S phase border [reviewed in (Bouchard & Schneider, 2004)]. Overall, a decreased ability to repair DNA damage caused by the interaction of HBx with the UVDDB complex, combined with modulation of cell proliferation pathways, could be important factors in the development of HBV-associated HCC.

3.4.2 HBx and p53

The results of various studies suggest that HBx may interact with the tumor suppressor p53 both *in vivo* and *in vitro*. HBx that was tagged with glutathione S-transferase (GST-HBx) was shown to interact with *in vitro* translated p53; a reciprocal interaction between GST-p53 and *in vitro* translated HBx was also observed (Wang et al, 1994). p53 and HBx produced by *in vitro* translation and subsequently co-immunoprecipitated with either anti-HBx or anti-p53 antibodies also confirmed an interaction between p53 and HBx (Feitelson et al, 1993). Several reports indicate that HBx can alter the sequence-specific DNA binding and transcriptional regulatory capacity of p53 as well as p53 stimulation of apoptotic pathways (Elmore et al, 1997; Lee & Rho, 2000; Wang et al, 1994; Wang et al, 1995). The results of one study suggested that HBx can interact with the carboxy-terminal domain of p53 and that this interaction leads to inhibition of p53-induced apoptosis in normal primary human fibroblasts (Wang et al, 1995). Results of another study suggested that p53 can be sequestered in the cytoplasm by HBx, thus leading to the inhibition of p53-mediated apoptosis (Elmore et al, 1997). Interestingly, there are varying results regarding the role of p53 in the regulation of apoptosis by HBx; both p53-dependent (Wang et al, 2008) and p53-independent regulation of apoptosis by HBx have been observed (Shintani et al, 1999; Terradillos et al, 1998). Studies have also interrogated the influence of p53 on HBx regulation of the cyclin-dependent kinase inhibitor, p21; HBx increased the level of p21 in NIH3T3 cells in the presence of p53 but did not increase the level of p21 when p53 was knocked down (Ahn et al, 2002). However, in a different study, HBx increased p21 levels in Hep3B cells, a p53 mutant HCC cell line (Park et al, 2000). Since activities of HBx that were linked to p53-dependent pathways were also observed in the absence of p53, the functional

importance of the putative interaction between p53 and HBx has been questioned (Groisman et al, 1999; Terradillos et al, 1998). In one study, an *in vivo* interaction between HBx and p53 was observed in liver tissues of HBV-infected patients with HCC; however, contradictory results were obtained in a different study where p53 was found to be present only in the nucleus and HBx predominantly in the cytoplasm of liver tissues of HBV-infected patients (Feitelson et al, 1993; Su et al, 2000). The effect of HBx on the activities of p53 could directly influence the development of HBV-associated HCC; deregulation of p53 activities, such as its ability to regulate the cell cycle and apoptotic pathways, could favor processes that cause hepatocyte transformation. Overall, studies that have addressed a potential interaction between HBx and p53 suggest that this may occur in a cell-specific context; additional studies are required to examine whether HBx and p53 interact in the context of HBV replication in normal hepatocytes.

3.5 HBx activates transcription

Although HBx does not bind directly to DNA, it can function as a modest transcriptional activator [reviewed in (Benhenda et al, 2009; Bouchard & Schneider, 2004; Koike, 2009)]. HBx can activate multiple viral and cellular transcription promoters and enhancers [reviewed in (Benhenda et al, 2009; Murakami, 2001; Yen, 1996)]. HBx can activate promoters that contain DNA binding sites for transcription factors such as nuclear factor-kappa-B (NF-κB), activating protein-1 (AP-1), nuclear factor of activated T cells (NFAT), cAMP response element-binding transcription factor (CREB)/activating transcription factor (ATF)-2, CCAAT/enhancer binding protein (C/EBP), and stimulating protein 1 (Sp1), to name a few (Carretero et al, 2002; Chirillo et al, 1996; Cougot et al, 2007; Doria et al, 1995; Lara-Pezzi et al, 1999; Lee et al, 1998; Lucito & Schneider, 1992; Maguire et al, 1991; Mahe et al, 1991; Natoli et al, 1994a; Purcell et al, 2001; Spandau & C., 1988; Su & Schneider, 1996; Waris et al, 2001; Williams & Andrisani, 1995). HBx can activate transcription factors directly, through interactions with specific transcription regulatory proteins, or indirectly, by activation of cytosolic signal transduction pathways [(reviewed in (Bouchard & Schneider, 2004)]. HBx can directly interact with components of the basal transcription machinery, such as TBP, RBP5, TFIIB, and TFIIH; in addition, it also interacts with CREB and c-AMP dependent transcription factor (ATF)-3, a member of the basic leucine zipper (b-Zip) family of transcription factors (Aufiero & Schneider, 1990; Barnabas et al, 1997; Cheong et al, 1995; Cougot et al, 2007; Haviv et al, 1998a; Haviv et al, 1998b; Lin et al, 1997; Maguire et al, 1991; Williams & Andrisani, 1995; Yang et al, 1999). Interestingly, some studies suggest that HBx can activate its own promoter through an X responsive element (XRE) and can also activate HBV enhancers (Doria et al, 1995; Faktor & Shaul, 1990; Spandau & C., 1988). Whether the transcriptional activation functions of HBx in normal hepatocytes and in the context of HBV replication completely overlap with activities identified in established cell lines awaits a comprehensive analysis of HBx activities during a natural HBV infection.

3.6 HBx activates multiple signal transduction pathways

HBx can regulate numerous signal transduction pathways, including the Ras-Raf-Mitogen activated protein kinase (MAPK) pathway, the Janus-kinase signal transducer and activator of transcription pathway (JAK-STAT pathway), the phosphoinositide 3-kinase (PI3K) pathway, the stress-activated protein kinase/NH2-terminal-Jun kinase (SAPK/JNK)

pathway, the transforming growth factor-β (TGF-β) pathway, the protein kinase C (PKC) and the p38 mitogen-activated protein kinase (p38MAPK) pathway (Benn & Schneider, 1994; Benn et al, 1996; Bouchard et al, 2003; Bouchard et al, 2001b; Chung et al, 2004; Cong et al, 1997; Cross et al, 1993; Johnson et al, 2000; Kekule et al, 1993; Klein et al, 1999; Klein & Schneider, 1997; Lara-Pezzi et al, 1999; Lee & Yun, 1998; Lucito & Schneider, 1992; Natoli et al, 1994a; Natoli et al, 1994b; Purcell et al, 2001; Tarn et al, 1999; Tarn et al, 2001; Tarn et al, 2002; Wang et al, 1998). The results of various studies suggest that HBx regulation of cytosolic signal transduction pathways could play a role in activation of transcription factors, HBx modulation of HBV replication, and HBx modulation of apoptotic and cell proliferation pathways [reviewed in (Benhenda et al, 2009; Bouchard & Schneider, 2004)].

The various signaling pathways that are stimulated by HBx have also been linked to mechanisms that are associated with cell transformation. For example, the Ras-Raf-MAPK signaling cascade has been implicated in the regulation of cell cycle progression, cellular differentiation, and cellular transcription pathways [reviewed in (Boguski & McCormick, 1993; McCubrey et al, 2007)]. HBx can activate the Ras/Raf kinase pathway in HeLa cells, a human cervical carcinoma cell line, and F9 cells, a mouse teratocarcinoma cell line; dominant negative mutants of Ras and the kinase Raf, an effector of the Ras-Raf-MAPK signaling cascade, blocked HBx activation of AP-1 (Natoli et al, 1994b). HBx activation of the Ras-Raf-MAPK signaling cascade was also observed in Chang cells, a human liver cell line, and directly in mouse livers (Benn & Schneider, 1994; Nijhara et al, 2001). Chang cells that expressed HBx had increased levels of Ras-GTP as compared to control cells, suggesting that HBx can increase the exchange of GDP for GTP (Benn & Schneider, 1994). Various reports indicate that the activation of Ras by HBx is indirect; HBx was not associated with Ras, Ras-GAP, the GTP exchange factor sons of sevenless (Sos), or the Ras adapter proteins Grb2 or Shc (Benn et al, 1996; Klein & Schneider, 1997). Instead, HBx was shown to constitutively activate non-receptor tyrosine kinases of the Src family (Src and Fyn), which can signal to Ras; inhibition of Src in Chang cells blocked HBx-induced Ras signaling and activation of the MAPK, ERK-2 (Klein et al, 1999; Klein & Schneider, 1997). HBx activation of Src kinases has been linked to the HBx-induced elevation of cytosolic calcium levels and activation of Pyk2 and FAK (Bouchard et al, 2006; Bouchard et al, 2001b). HBx stimulation of Src kinases promoted cycling of growth-arrested HepG2 and Chang cells; HBx induced arrested cells to exit G0 but stall at the G1/S border of the cell cycle, an activity that is thought to be important for HBV replication (Bouchard et al, 2001a). Importantly, active Src kinases are required for WHV replication, and HBx activation of Pyk2 and FAK, which is required for HBx activation of Src kinases, is also important for HBV replication (Bouchard et al, 2006; Bouchard et al, 2001b; Klein et al, 1999; Klein & Schneider, 1997). Finally, HBx-induced activation of Src kinases has been shown to be required for the stimulation of p38 MAPK, STAT3, and AP-1; activation of these proteins have been linked to various cell transformation processes and could contribute to processes that link chronic HBV infections to HCC development (Klein et al, 1999; Klein & Schneider, 1997; Lee & Yun, 1998; Tarn et al, 2002).

3.7 HBx regulates apoptotic pathways

Whether HBx is anti- or pro-apoptotic has been the subject of considerable debate. HBx has been reported to induce apoptosis (Bergametti et al, 1999; Chami et al, 2003; Chirillo et al,

1997; Kim et al, 1998; Kim et al, 2008; Kim & Seong, 2003; Koike et al, 1998; Lee et al, 2004; Liang et al, 2007; Liu et al, 2007; Lu & Chen, 2005; Miao et al, 2006; Shintani et al, 1999; Su & Schneider, 1997; Su et al, 2001; Takada et al, 1999; Tanaka et al, 2004; Terradillos et al, 2002; Terradillos et al, 1998; Wang et al, 2004a), sensitize cells to pro-apoptotic stimuli (Lee et al, 2004; Su & Schneider, 1997; Wang et al, 2004a), inhibit apoptosis (Clippinger et al, 2009; Diao et al, 2001; Elmore et al, 1997; Gottlob et al, 1998; Kang-Park et al, 2006; Lee et al, 2001; Pan et al, 2001; Shih et al, 2000), or have no impact on apoptosis (Klein et al, 2003; Madden et al, 2000; Yun et al, 2002). Interestingly, the effect of HBx on apoptosis was found to differ depending on the differentiation status of hepatocytes; HBx sensitized dedifferentiated hepatocytes, but not differentiated hepatocytes, to apoptotic signals (Wang et al, 2004a). The differing effects of HBx expression on apoptotic pathways that have been reported are likely attributed to the use of numerous cell types and experimental conditions for studying HBx apoptotic activities, and it is now apparent that HBx activities can vary in different cellular environments. Few studies have analyzed the impact of HBx expression on apoptosis in the context of HBV replication. Recent studies in cultured primary rat hepatocytes demonstrated that HBx has both pro- and anti-apoptotic activities in normal hepatocytes; similar HBx effects were observed when HBx was expressed in the absence of other HBV proteins or in the context of HBV replication. The anti-apoptotic activity of HBx in cultured primary rat hepatocytes was linked to HBx activation of NF-κB; however, when activation of NF-κB was blocked, HBx was found to induce apoptosis through a mechanism that was dependent on activities of the MPTP (Clippinger et al, 2009). The exact nature of HBx regulation of the MPTP is currently unknown. Whether HBx regulates apoptosis to affect HBV replication or the spread of HBV within the infected liver is not known. It is possible that HBx inhibits apoptosis during early stages of an HBV infection and later induces apoptosis to assist in viral spread [reviewed in (Benhenda et al, 2009; Ng & Lee, 2011)]. Although the pro-apoptotic effects of HBx might facilitate HBV replication and spread and lead to evasion of host cell-mediated immunity [reviewed in (Arbuthnot et al, 2000)], a recent study has provided evidence that modulation of apoptosis during an HBV infection is unlikely to affect viral spread (Arzberger et al, 2010). It is possible that the ability of HBx to induce or inhibit apoptosis might change during the course of an HBV infection as the liver is undergoing regeneration, or when the hepatocytes undergo transformation or respond to cytokines such as TNFα [reviewed in (Benhenda et al, 2009; Brenner, 1998; FitzGerald et al, 1995)]; both the activation and inactivation of apoptosis by HBx could contribute to HCC development. Enhanced compensatory hepatocyte regeneration that is induced by an HBx pro-apoptotic effect could lead to selection of hepatocytes that are resistant to apoptotic signals, thus leading to HCC (Koike et al, 1998). Alternatively, inhibition of apoptosis by HBx may increase the accumulation of potentially transforming mutations, leading to the development of liver cancer [reviewed in (Arbuthnot et al, 2000)]. Although the precise mechanisms that underlie HBx regulation of apoptosis in naturally infected hepatocytes remain incompletely understood, HBx modulation of hepatocyte apoptotic pathways is a potential mechanism that could influence the development of HBV-associated HCC.

4. HBx and the cell cycle

4.1 Cell cycle overview

The process of cellular replication and division is known as the cell cycle. The cell cycle is a highly regulated series of events and can be controlled by intracellular and extracellular

factors [reviewed in (Harper & Brooks, 2005)]. Differentiated cells, such as hepatocytes, are typically maintained in a non-dividing, resting state, known as quiescence [reviewed in (Taub, 2004)]. When cells are quiescent, also known as the G0 phase, they must receive a signal in order to exit the G0 phase and enter the cell cycle [reviewed in (Cook et al, 2000; Harper & Brooks, 2005; Vermeulen et al, 2003)]. If they receive this signal, they will enter into the first phase of the cell cycle, known as the Gap 1 (G1) phase. During G1 phase, cells are preparing to replicate their DNA, and as long as the first major checkpoint of the cell cycle, the restriction point (R) at the G1/S border, is not activated and the growth signal is still present, cells will proceed into the Synthesis (S) phase. During S phase, the cells undergo DNA replication; once cells enter into this phase, DNA replication is completed regardless of removal of the growth signal or the presence of DNA damage. After DNA replication is completed, cells enter into the Gap 2 (G2) phase where the cellular machinery checks for DNA damage that may have accumulated during DNA replication and prepares for the Mitosis (M) phase. Once the proper signals are in place for cell cycle progression, cells will enter into M phase, during which mitosis occurs [reviewed in (Harper & Brooks, 2005; Vermeulen et al, 2003)]. A third checkpoint, the spindle checkpoint, exists after metaphase and prior to anaphase; at this checkpoint the cell employs methods to detect improper alignment of chromosomes on the mitotic spindle (Amon, 1999; Fang et al, 1998). If improper alignment is not detected, cells will continue into anaphase, at which point, the cells will complete the cell cycle, generating two daughter cells [reviewed in (Vermeulen et al, 2003)].

4.1.1 Positive regulators of cell cycle progression: Cyclins and cyclin-dependent kinases

Transition from one phase of the cell cycle to the next is tightly regulated and progressive. Key regulatory proteins that control cell cycle progression are cyclins and cyclin-dependent kinases (CDKs). CDKs are a family of serine/threonine protein kinases that are only activated at certain points in the cell cycle, although their expression levels remain constant throughout the entire cell cycle [reviewed in (Vermeulen et al, 2003)]. Currently, there are five CDKs that are associated with cell cycle progression in mammalian cells: CDKs 4 and 6, which are active during early G1 phase, CDK2, which is active in late G1 and S phase, CDK1, which is active during G2 and M phase, and CDK7, which can act in combination with cyclin H as a CDK-activating kinase (CAK) [(Fisher & Morgan, 1994); reviewed in (Harper & Brooks, 2005)]. CDK activity is highly regulated and requires both the expression of activating proteins, cyclins, and phosphorylation of the cyclin-CDK complex [reviewed in (Harper & Brooks, 2005; Vermeulen et al, 2003)]. The expression of cyclins is highly regulated, and their levels rise and fall depending on the cell cycle phase (Evans et al, 1983; Pines & Hunter, 1991). There are three D type cyclins, cyclin D1, cyclin D2, and cyclin D3, which bind to CDK4 and CDK6, resulting in activation of these CDKs; activation of CDK4 and CDK6 is required for entry into the cell cycle [reviewed in (Sherr, 1993; Sherr, 1994)]. Unlike the other cyclins, cyclin D expression is maintained as long as growth factor stimulation is present (Assoian & Zhu, 1997). Cyclin E binds to CDK2 and regulates cell cycle progression from G1 into S phase (Ohtsubo et al, 1995). Cyclin A binds to CDK2 to regulate S phase progression and also binds to CDK1 during late G2 and M phase to promote entry into M phase (Arellano & Moreno, 1997; Girard et al, 1991; King et al, 1994;

Walker & Maller, 1991). During mitosis, an additional cyclin, cyclin B, is expressed; cyclin B binds to CDK1 to regulate the remainder of mitosis (Arellano & Moreno, 1997; King et al, 1994). In accordance with the dynamic nature of the induction of cyclin expression, cyclins are rapidly degraded when the cell cycle has progressed beyond the phase during which their expression is required. Cyclins D and E contain a PEST sequence, a segment rich in proline (P), glutamic acid (E), serine (S), and threonine (T) residues, and cyclins A and B contain destruction boxes. The PEST sequence and destruction boxes facilitate ubiquitin-mediated proteasomal degradation of the cyclins (Glotzer et al, 1991; Rechsteiner & Rogers, 1996).

Full CDK activity is dependent upon proper cyclin expression and binding and phosphorylation of the cyclin-CDK complex. Phosphorylation of the cyclin-CDK complex occurs on conserved threonine and tyrosine residues and induces conformational changes, which can enhance cyclin binding and CDK activity (Jeffrey et al, 1995; Paulovich & Hartwell, 1995). Activation of CDK4 requires phosphorylation of threonine 172, activation of CDK2 requires phosphorylation of threonine 160, and activation of CDK1 requires phosphorylation of threonine 161 by the CDK7-cyclin H complex, CAK [reviewed in (Vermeulen et al, 2003)]. Alternatively, phosphorylation of cyclin-CDK complexes can also inhibit CDK activity when the cyclin-CDK complex is primed by cyclin binding to its partner CDK, but the cell does not yet require full activation of the CDK. For example, the cyclin A-CDK1 complex can be inhibited by phosphorylation at tyrosine 15 and/or threonine 14 by the kinases Wee1 and Myt1. The inhibitory phosphate can be removed by the Cdc25 phosphatase; this dephosphorylation is required for the full activation of CDK1 and subsequent progression through the cell cycle (Lew & Kornbluth, 1996). Once fully activated, the CDKs induce downstream signaling events by phosphorylating select substrates that regulate cell cycle progression (Morgan, 1997; Pines & Hunter, 1991). One such event is the phosphorylation of the retinoblastoma tumor suppressor gene (Rb) by the CDK4/6-cyclin D complex. In its active, dephosphorylated state, Rb is in a complex with the histone deacetylase protein HDAC and the transcription factors E2F-1 and DP-1. Upon phosphorylation in G1 phase, Rb is inactivated, resulting in the release of E2F-1 and DP-1, which then activate transcription of genes which are required for S phase progression, including those encoding cyclin E, cyclin A, and Cdc25 (Brehm et al, 1998; Buchkovich et al, 1989; Kato et al, 1993). Rb remains hyperphosphorylated for the remainder of the cell cycle; the cyclin E-CDK2 complex stabilizes this hyperphosphorylated state. The cyclin E-CDK2 complex can also phosphorylate its negative regulator p27, resulting in proteasomal degradation of p27 (Hinds et al, 1992; Montagnoli et al, 1999).

4.1.2 Negative regulators of cell cycle progression: CDK inhibitors

CDK activity can also be negatively regulated by interaction with various cellular proteins that are generically named CDK inhibitors, or CKIs. CKIs can bind to isolated CDKs or to the CDK-cyclin complex to prevent the full activation of CDKs [reviewed in (Harper & Brooks, 2005)]. There are two families of CKIs, the INK4 (inhibitor of CDK4) family and the Cip (CDK-interacting protein)/Kip (kinase inhibitor protein) family [reviewed in (Sherr & Roberts, 1995)]. The INK4 family includes p15 (INK4b), p16 (INK4a), p18 (INK4c), and p19 (INK4d) [reviewed in (Carnero & Hannon, 1998)]. Members of the INK4 family inactivate CDK4 and CDK6 by forming stable complexes with the CDK prior to cyclin binding; this blocks association of CDK4 and CDK6 with cyclin D and prevents entry into G1 phase

[reviewed in (Harper & Brooks, 2005)]. Members of the Cip/Kip family include p21 (Waf1, Cip1), p27 (Cip2), and p57 (Kip2); these proteins contain a conserved region that is involved in cyclin binding and kinase inhibition [(Polyak et al, 1994a; Polyak et al, 1994b); reviewed in (Harper & Brooks, 2005; Roberts et al, 1994)]. Unlike members of the INK4 family, members of the Cip/Kip family bind to and inhibit the activity of the entire cyclin-CDK complex. Additionally, this CKI family displays a broader specificity than the INK4 family, as its members can bind and inhibit the activities of many cyclin-CDK complexes, including cyclin E-CDK2, cyclin A- CDK2, and cyclin B-CDK1 [(Hengst et al, 1998; Hengst & Reed, 1998); reviewed in (Harper & Brooks, 2005)]. Interestingly, the Cip/Kip family of CKI proteins can coordinate the assembly of the cyclin D-CDK4/6 complexes in the early G1 phase and stabilize this complex throughout G1 [(Cheng et al, 1999); reviewed in (Sherr & Roberts, 1999)].

4.2 The cell cycle and HCC

Deregulation of the cell cycle and alteration in the expression of cyclins and the activities of CDKs are frequently observed in transformed cells; consequently, disruption of normal mechanisms that regulate the cell cycle is thought to contribute to the development of many cancers [reviewed in (Hanahan & Weinberg, 2000)]. The expression of many cell cycle regulatory proteins is altered during the onset of HCC and is continually deregulated throughout the progression of liver cancer. For example, the transcription promoters of the *p15* and *p16* genes are frequently hypermethylated (Csepregi et al, 2010; Matsuda et al, 1999; Shim et al, 2003a; Shim et al, 2003b; Wong et al, 2000a; Wong et al, 2000b) and a significant percentage of HCCs are associated with decreased expression of p16, which occurs via hypermethylation, deletion, or mutation of the *p16* gene (Biden et al, 1997; Chaubert et al, 1997; Csepregi et al, 2010; Hui et al, 1996; Liew et al, 1999; Matsuda et al, 1999; Shen et al, 1998). Expression of DNA methyltransferase I, which methylates the *p16* promoter to inhibit transcription, is frequently upregulated in HCC (Huang et al, 2010; Oh et al, 2007). Decreased expression of p18, a potential tumor suppressor, is associated with poorly differentiated HCCs (Morishita et al, 2004). Interestingly, an increase in the phosphorylation of Rb has also been observed in HCCs that were negative for p18 expression (Morishita et al, 2004). Rb, a known tumor suppressor, is frequently inactivated (hyperphosphorylated) in HCC; approximately 15-30% of advanced HCCs display mutations in Rb [reviewed in (Burkhart & Sage, 2008)]. In addition to alterations in the INK4 family of CKIs, variations in the expression levels of the Cip/Kip family have also been found in HCC. Although a number of studies have demonstrated that the downregulation of p21 and p27 occurs in HCC, some other reports have suggested that the expression of p21 and p27 is increased in HCC (Gramantieri et al, 2003; Qin & Ng, 2001; Ren, 1991). Overexpression of cyclin D1 in mice has been linked to the development of hyperplasia and ultimately liver cancer (Deane et al, 2001). Importantly, the tumor suppressor p53 is frequently lost or mutated in many cancers, including in liver tumors [reviewed in (Hollstein et al, 1991)]. It is possible that p53 mutations could lead to the alteration of the cell cycle profile of hepatocytes and contribute to the development of liver cancer. Overall, it is evident that alterations in the expression levels or activities of cell cycle regulatory proteins and protein complexes could play a major role in hepatocarcinogenesis.

4.3 HBV replication and the cell cycle

DNA viruses have evolved different strategies to deregulate cell cycle checkpoint controls and modulate cell proliferation pathways. Because many DNA viruses primarily infect

differentiated, quiescent cells, it is thought these viruses must induce cells to exit G0 and enter the cell cycle to create an environment that generates factors, such as nucleotides, that are required for viral replication [reviewed in (Mukherji & Kumar, 2008; Swanton & Jones, 2001)]. An unfortunate consequence of virus-mediated alterations in normal cell cycle control mechanisms is that these viral effects may ultimately generate an environment that also favors cell transformation and cancer development [reviewed in (Bouchard & Navas-Martin, 2011; Mukherji & Kumar, 2008)].

HBV replication has been associated with modulation of cell cycle progression, and HBV replication has been shown to be cell cycle dependent in certain experimental systems [reviewed in (Madden & Slagle, 2001)]. HBV replication in Huh7 cells, a human hepatoma cell line, and in primary marmoset hepatocytes, was shown to regulate the cell cycle (Chin et al, 2007). Huh7 cells infected with HBV were found to stall in the G2 phase of the cell cycle; HBV replication in these cells increased activation of both MAPK and Akt pathways. Interestingly, p53, p21, cdc2 and intranuclear cyclin B1 levels were elevated in primary marmoset hepatocytes that were infected with HBV, which also suggested features consistent with a G2 arrest (Chin et al, 2007). Alternatively, the results of studies in HepG2.2.15 cells, human hepatoblastoma HepG2 cells that contain an integrated HBV genome and replicating HBV, and Huh7 cells that were transiently transfected with the HBV genome showed that expression of the HBV genome caused these cells to progress through the G1 phase while inhibiting entry into S phase (Friedrich et al, 2005). The results of a different study in HepG2.2.15 cells also showed decreased proliferation of HepG2.2.15 cells as compared to HepG2 cells; this study also demonstrated that HBV can modulate the expression levels of cell cycle regulatory proteins, which resulted in a G1 phase arrest (Wang et al, 2011). Cumulatively, it can be concluded that HBV infection can influence cell cycle progression; however, the exact consequence of HBV infection on cell proliferation pathways is likely influenced by the characteristics of the cell type used for a particular study.

Several studies have analyzed the impact of the cell cycle phase on HBV replication. HBV replication in HepG2.2.15 cells varied depending on the phase of the cell cycle. Arresting HepG2.2.15 cells in either G1 or G2 resulted in an increase in the levels of HBV DNA and mRNA, while cell entry into S phase increased cellular DNA synthesis and decreased levels of HBV replication (Huang et al, 2004; Ozer et al, 1996). Studies in liver specimens from HBV-infected patients demonstrated that hepatocytes expressing proliferating cell nuclear antigen (PCNA), which is expressed during S phase and required for DNA replication [reviewed in (Harper & Brooks, 2005)], contained negligible amounts of HBV-specific DNA (Ozer et al, 1996). Cumulatively, the results of these studies suggest that HBV replication is decreased when cells are actively proliferating. The results from a recent study suggested, although did not definitively show, that microRNA (miRNA) regulation of cell proliferation pathways could affect HBV replication; over-expression of miRNA-1 (miR-1) arrested HepG2.2.15 cells in the G1 phase and promoted differentiation, which could be beneficial for HBV replication (Zhang et al, 2011). In contrast to studies that demonstrated that HBV replication is affected by the phase of the cell cycle, one group demonstrated that HBV replication is independent of cell cycle status in HBV-transgenic mice (Guidotti et al, 1997). However, because HBV-transgenic mice do not produce cccDNA and do not recapitulate every step of an authentic HBV infection, it is unclear whether results in this system accurately reflect all mechanisms that can regulate HBV replication (Guidotti et al, 1995).

Overall, the results of most studies suggest that cell proliferation pathways are altered in cells with replicating HBV and that the status of the cell cycle can influence HBV replication. The effect of cell cycle phase on HBV replication in cultured primary rat and human hepatocytes will be discussed in a separate section of this chapter.

4.4 HBx modulation of cell cycle

HBx expression has been linked to modulation of cell cycle progression, although the effect of HBx on cell proliferation pathways has varied depending on the cell type used and the experimental conditions of the study [reviewed in (Madden & Slagle, 2001)]. In this section, we summarize published studies that have analyzed the impact of HBx on cell cycle progression in immortalized or transformed cells; more recent studies in cultured primary hepatocytes will be discussed in a separate section. Overall, studies in various immortalized or transformed cells have shown that HBx can induce cells to enter the cell cycle, enter the cell cycle but stall at G1/S border of the cell cycle, or progress more rapidly through the cell cycle (Benn & Schneider, 1994; Benn & Schneider, 1995; Bouchard et al, 2001a; Chen et al, 2008; Koike et al, 1994; Lee et al, 2002; Mukherji et al, 2007; Singh et al, 2011; Zhang et al, 2005). It is likely that the observed variations in HBx effects reflect the use of different cell lines, different methods of HBx expression, and whether or not the studies were performed in the presence of growth factors. It is important to note that some seemingly discrepant results that have been reported may reflect a different interpretation of similar data. For example, HBx expression can cause cells in G0 to exit G0 but stall at the G1/S phase; this could be interpreted as stimulation of cell progression beyond G0 or inhibition of cell progression into S phase [reviewed in (Bouchard & Schneider, 2004)]. Additionally, most of the studies that analyzed the impact of HBx on cell cycle proliferation and cell cycle regulatory proteins were conducted when HBx was expressed in the absence of other HBV proteins, and it is unclear whether all the reported HBx activities that were associated with regulation of cell proliferation pathways are also present in the context of HBV replication.

Serum deprivation of cultured cells is a commonly used method for arresting cells in G0 or at the G0-G1 junction. When serum-starved Chang liver cells were infected with HBx-expressing recombinant adenoviruses, HBx increased cellular DNA synthesis and stimulated cell proliferation (Benn & Schneider, 1994). HBx eliminated the requirement for serum in cell cycle activation in Chang cells, and HBx-expressing Chang cells that were stimulated with serum entered S phase and progressed through the G2/M checkpoint more rapidly than control cells (Benn & Schneider, 1995). Additionally, HBx activation of Ras was necessary for HBx-dependent activation of cell cycling in Chang cells (Benn & Schneider, 1995). HBx activation of Src kinases was linked with the ability of HBx to cause serum-starved, quiescent Chang cells to transit through the G1 phase but stall at the G1/S phase of the cell cycle (Bouchard et al, 2001a). HBx expression in HepG2 cells increased cellular proliferation, as demonstrated in a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay (Zhang et al, 2005). Furthermore, an increase in DNA synthesis, as demonstrated by bromo-deoxyuridine (BrdU) incorporation, was observed in serum starved, HBx-expressing NIH3T3 cells, a mouse embryonic fibroblast cell line (Koike et al, 1994). Additional analysis of cell cycle progression in NIH3T3 cells indicated that expression of HBx caused cells to move from the G0/G1 phase to the S and G2/M phase (Koike et al, 1994). Alternatively, studies in HL-7702 cells, a normal human liver cell line, showed that the expression of HBx in these cells caused an increased accumulation of cells

in S phase (Chen et al, 2008). The results from another study in Huh7 and human embryonic kidney 293 (HEK 293) cells suggested that HBx can cause these cells to transit through the G0 and G1/S checkpoints of the cell cycle even in the absence of serum (Mukherji et al, 2007). Additionally, HBx expression was found to increase the rate of entry of cells into S phase, thus causing rapid cycling of these cells; constitutive activation of Src kinases was shown to be required for HBx-mediated shortening of the cell cycle (Mukherji et al, 2007). In a seminal study that may explain the reason for the different effects of HBx on the cell cycle that have been observed in various cellular contexts, HBx expression was shown to modulate cell cycle progression differently in cells that had characteristics of differentiated or dedifferentiated hepatocytes. In these studies, two HBx-expressing cell lines were derived from the same parental AML12 liver cell line. AML12 cells are immortalized mouse hepatocytes that were originally derived from TGFα-transgenic mice; these cells maintain many characteristics that are similar to normal hepatocytes in the liver (Wu et al, 1994a). One of the newly derived HBx-expressing AML12 cell lines showed characteristics similar to differentiated hepatocytes, whereas the other cell line displayed a more dedifferentiated phenotype (Tarn et al, 1999; Wu et al, 1994b). HBx expression in the more differentiated cells caused the cells to rapidly progress through the cell cycle; however, HBx expression in the more dedifferentiated cells caused these cells to enter the cell cycle but pause in S phase (Lee et al, 2002). These observations support the notion that the impact of HBx on cell proliferation pathways varies depending on the characteristics of the cells used for the study. Taken together, the results of these numerous studies suggest that HBx can modulate cell cycle progression, though the exact impact of HBx on cell proliferation pathways when HBx is expressed from replicating HBV in normal hepatocytes in the liver remains to be completely defined.

4.4.1 HBx regulation of cyclins and CDKs

The results of multiple studies have demonstrated that HBx can regulate the levels of cyclins and the activities of CDKs, although these HBx activities have varied in different experimental conditions. In one study, both stable and transient expression of HBx was found to increase the level of cyclin D1 in HepG2 cells (Jung et al, 2007). The level of cyclin D1 was also elevated in Huh7 cells that contained replicating HBV; however, this observation could not be directly linked to HBx since the effects of HBV on cell cycle regulatory proteins was not compared to an HBx-deficient HBV (Chin et al, 2007). Studies from another group suggested that HBx activation of NF-κB may play a role in up-regulation of cyclin D1 levels in Chang cells (Park et al, 2006). Additional studies have shown that HBx can also regulate the expression levels of cyclins that are required during later stages of the cell cycle. HBx activated the cyclin A promoter, increased cyclin A protein levels, and promoted the formation of cyclin A-CDK2 complexes in ts13 cells (Bouchard et al, 2001a); these cells are derived from a hamster cell line containing a temperature-sensitive defect in TBP-associated factor 250 (TAFII 250). The TAFII 250 ts13 mutation has been shown to induce cell cycle arrest and apoptosis when these cells are shifted from the permissive temperature to the nonpermissive temperature (Hayashida et al, 1994; Talavera & Basilico, 1977). The ability of HBx to activate the endogenous cyclin A promoter and cyclin A-CDK2 complexes was linked to stimulation of Src kinase signaling pathways (Bouchard et al, 2001a). HBV replication in Huh7 cells increased the levels of cyclin A and intranuclear cyclin B, which is active during the G2/M phase. Whether the elevation of

cyclin A or intranuclear cyclin B was caused by HBx in these studies is not entirely clear because the results were not compared to similar assays with an HBx-deficient HBV (Chin et al, 2007). HBx increased the activation of CDK2 and CDK1 in Chang liver cells; this HBx activity was blocked with a dominant-negative mutant of Ras (Benn & Schneider, 1995). Finally, the results of studies in Huh7 cells demonstrated that HBx expression increased the stability of cyclin E in these cells and significantly enhanced CDK2 activity (Mukherji et al, 2007). Moreover, HBx was able to bind to the cyclin E/A-CDK2 complex in Huh 7 cells; expression of c-terminal src kinase (CSK), a physiological inhibitor of Src kinases, abrogated the interaction of HBx with the cyclin E-CDK2 complex (Mukherji et al, 2007). Overall, the various studies in immortalized and transformed cells demonstrate that HBx can modulate the levels and activities of positive regulators of the cell cycle. Whether similar HBx activities are apparent in the context of an authentic HBV infection of hepatocytes in the liver awaits further investigation.

4.4.2 HBx regulation of CKIs

In addition to the influence of HBx on positive regulators of the cell cycle, the results of some studies have suggested that HBx can also affect negative cell cycle regulators. These studies have mainly focused on the effect of HBx expression on the expression levels of p16, p21, and p27. Studies in HBV-associated cirrhotic livers and HBV-producing HCC cell lines showed a correlation between the hypermethylation of the *p16* gene, decreased expression of p16, and the presence of HBV (Shim et al, 2003b). Although this study demonstrated that HBV replication may be associated with deregulation of p16, these findings were not directly linked to HBx expression. However, in a subsequent study in HBV-associated HCC liver sections, the methylation status of the *p16* promoter was analyzed; liver sections that contained high levels of methylated *p16* promoters also had high expression levels of HBx, indicating that HBx expression correlates with the methylation status of the *p16* promoter (Zhu et al, 2007). Studies in HepG2 cells stably transfected with an HBx-expression plasmid showed that HBx induced hypermethylation of the *p16* promoter and down-regulation of p16 protein levels, which resulted in the activation of the cyclin D1-CDK4/6 complex, phosphorylation of Rb, activation of E2F1, and transcriptional activation of DNA methyltransferase 1 (DNMT1) (Jung et al, 2007). Interestingly, this induction of DNMT1 expression was found to be required for the *p16* promoter methylation and also for the expression of DNMT1 itself, suggesting that there is communication between p16 and DNMT1 (Jung et al, 2007). The results of an additional study demonstrated that the HBx-mediated reduction in p16 expression enabled HBx-expressing HepG2 cells to evade H_2O_2-induced senescence (Kim et al, 2010). Studies in liver tissue samples from HBV-associated HCCs and corresponding HBV-infected non-cancerous liver sections showed that in the non-cancerous tissues, HBx expression correlated positively with DNMT1 expression and negatively with p16 protein expression (Zhu et al, 2010). Alternatively, in the HCC tissues, HBx expression still correlated positively with DNMT1 expression, but did not correlate with the hypermethylation of the *p16* promoter or with p16 expression, suggesting that HBx-mediated hypermethylation of *p16* may play a role in the early stages of HBV-related hepatocarcinogenesis (Zhu et al, 2010).

The results of various studies have also suggested that HBx can alter the expression of p21 and p27. In Huh7 cells that were infected with a recombinant HBV-encoding adenovirus, an

increase in p21 was observed; however, these findings were not directly linked to HBx expression because the results were not compared to Huh7 cells infected with a recombinant adenovirus that contained an HBV mutant that lacked HBx expression (Chin et al, 2007). In another study in HBx-expressing NIH3T3 cells, a dose-dependent increase in the transcription of p21 was observed when p53 was functional (Ahn et al, 2002). Alternatively, in the absence or down-regulation of p53, a repression of p21 transcription by HBx was observed in NIH3T3 cells (Ahn et al, 2002). Moreover, HBx expression decreased the level of p21 RNA in NIH3T3 cells, which may be due to p53-independent transcriptional repression, resulting from an HBx-mediated Sp-1 inactivation (Ahn et al, 2001). Sp-1 is a transcription factor that can activate transcription of many target genes, including p21 (Pardali et al, 2000). Transcriptional repression of p21 was also observed in NIH3T3 cells co-transfected with plasmids encoding HBx and the hepatitis C virus (HCV) core protein (Han et al, 2002). While there have been many studies that have investigated the role of HBx in regulating p21, much less is known about the effect of HBx on p27. In Huh7 cells, HBx expression was found to increase proteasomal degradation of p27 (Mukherji et al, 2007). Overall, the precise impact of HBx expression on members of the Cip/Kip family seems to vary in different cellular contexts, and both upregulation and downregulation of the CIP/KIP family has been observed. Interestingly, the results of one study showed that the level of HBx expression influences its effects on p21 and p27 (Leach et al, 2003). When HBx was expressed at low levels in Chinese hamster ovary (CHO) cells, an increased activity of the p21 and p27 promoters was observed. Alternatively, when HBx was expressed at high levels in CHO cells, there was inhibition of the activity of the p21 and p27 promoters. This study suggests that the experimental conditions that are used to study HBx modulation of CKIs can influence the observed effect of HBx.

4.4.3 HBx regulation of cell proliferation in primary hepatocytes

Although various studies have analyzed the impact of HBx on cell proliferation pathways, many of these studies have identified different HBx effects. Most of these studies were conducted in transformed or immortalized cell lines and when HBx was overexpressed in the absence of other HBV proteins, which could contribute to the different HBx activities that were observed. Because established cell lines often contain changes in pathways that control normal cell cycle progression, observations of HBx activities in these cells could reflect HBx activities that are apparent in a specific context but not necessarily present in normal hepatocytes during an HBV infection. Recent studies in cultured primary hepatocytes have begun to address the effect of HBx expression, both on its own and when expressed in the context of replicating HBV, on hepatocyte cell cycle regulatory pathways. These studies have provided important insights into HBx activities that regulate HBV replication and could influence the development of HBV associated HCC.

Cultured primary rat hepatocytes are a biologically relevant model system that can be used to characterize HBx activities in normal, untransformed hepatocytes. In a series of studies in cultured primary rat hepatocytes, the impact of HBx on cell cycle regulation and HBV replication in normal hepatocytes was analyzed (Figure 3). In cultured primary rat hepatocytes, HBx decreased the expression level of both p15 and p16 while increasing the expression of p21 and p27, demonstrating that HBx decreased the levels of factors that maintained the quiescent status of hepatocytes and elevated the levels of inhibitors of cell

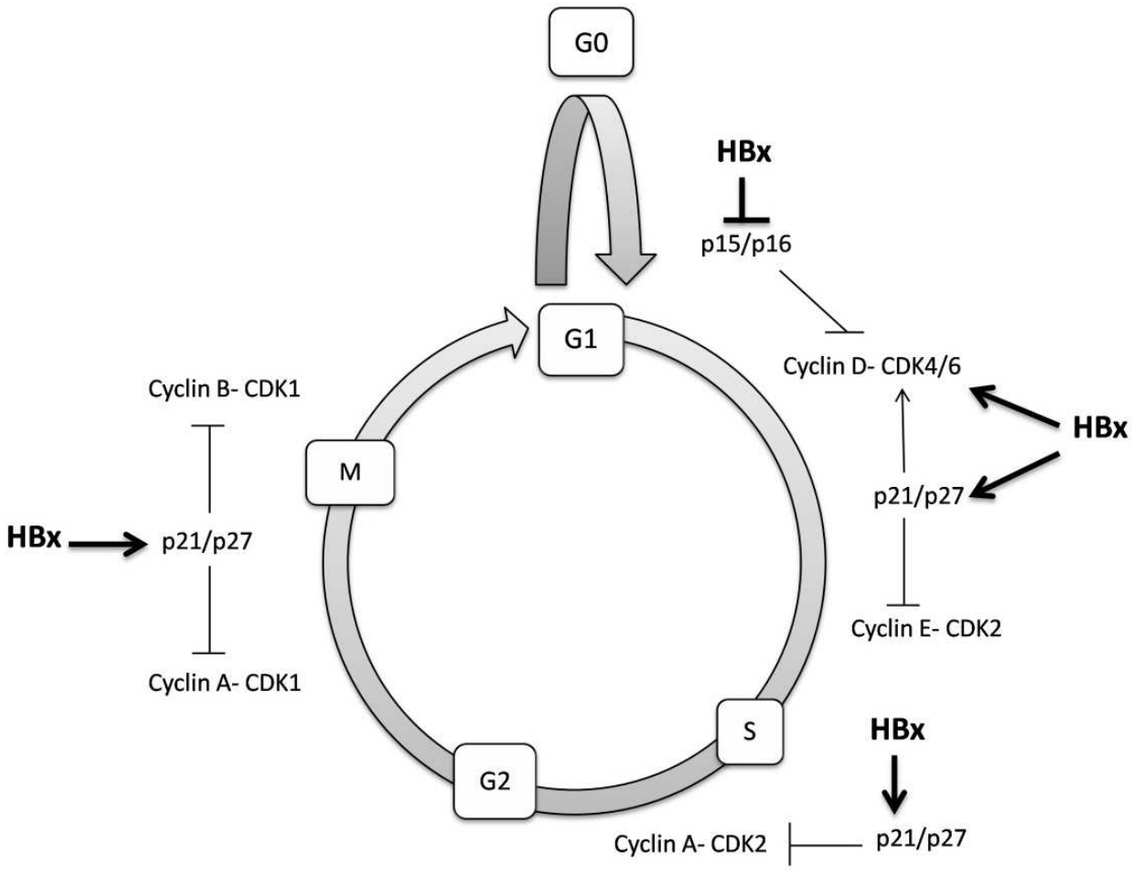

Fig. 3. Summary of HBx effects on the cell cycle in cultured primary hepatocytes. See text for details.

cycle progression past late G1 phase (Gearhart & Bouchard, 2010a). Similar studies in primary mouse hepatocytes that were infected with a recombinant HBx-expressing adenovirus also showed that HBx increased expression of both p21 and p27 and decreased DNA synthesis (Qiao et al, 2001). Finally, another study also identified an increase in p21 protein levels in primary mouse hepatocytes infected with a recombinant HBV-expressing adenovirus; however, these results were not directly linked to HBx expression because they were not compared to similar studies with an HBx-deficient HBV (Chin et al, 2007). In cultured primary rat hepatocytes, an increase in cyclin D1 and cyclin E expression in the presence of HBx was also observed; however, HBx-induced changes in S phase activating proteins, such as cyclin A and PCNA, were not observed, indicating that HBx expression did not cause entry into S phase (Gearhart & Bouchard, 2010a). Importantly, similar HBx activities were observed in cultured primary human hepatocytes that were infected with a recombinant HBx-expressing adenovirus (Gearhart & Bouchard, 2011) and in cultured primary rat hepatocytes infected with recombinant adenoviruses encoding the full HBV genome that was compared to a mutant HBV that did not produce HBx (Gearhart & Bouchard, 2010a). To determine the significance of the HBx-mediated elevation of cyclin D1 and cyclin E protein levels, the effect of HBx on the activities of CDK4 and CDK2 in cultured primary rat hepatocytes was also examined (Gearhart & Bouchard, 2010a). CDK4 and CDK2

activities were analyzed using standard kinase assays; the substrate for CDK4 phosphorylation was Rb and the substrate for CDK2 phosphorylation was histone H1. To analyze CDK2 activity, primary rat hepatocytes were plated at 50% confluency and stimulated with hepatocyte growth factor (HGF) to induce proliferation (Gearhart & Bouchard, 2010a). HBx, expressed either alone or in the context of HBV replication, increased the activity of CDK4 but decreased the activity of CDK2 (Gearhart & Bouchard, 2010a). The results of these studies confirmed that the HBx-mediated increase in cyclin D1 correlated with increased CDK4 activity; however, the increase in cyclin E expression was not coupled to an increase in CDK2 activity, confirming that HBx prevents cell cycle progression past G1 phase in normal hepatocytes. It is likely that HBx elevation of p21 and p27 inhibits the activity of cyclinE-CDK2 complexes. In contrast, the CIP/KIP proteins are thought to stimulate cell cycle progression in early G1 phase, which may contribute to the ability of HBx to cause hepatocytes to exit G0 and enter G1 [(Cheng et al, 1999); reviewed in (Harper & Brooks, 2005; Sherr & Roberts, 1999)]. Overall, these studies suggest that HBx causes quiescent hepatocytes to exit G0 but stall in G1.

Studies in primary rat hepatocytes were also conducted to examine the effect of cell cycle progression on HBV replication. Downregulation of p16 is required for cells to exit quiescence and enter the cell cycle [reviewed in (Harper & Brooks, 2005)]; primary rat hepatocytes were transfected with a p16 overexpression plasmid, infected with an HBV-encoding recombinant adenovirus, and HBV replication was analyzed to determine if HBV replication requires quiescent hepatocytes to enter the cell cycle (Gearhart & Bouchard, 2010a). Interestingly, preventing hepatocytes from entering the cell cycle by the overexpression of p16, inhibited HBV replication (Gearhart & Bouchard, 2010a). Importantly, this study was the first to directly examine the effect of cell quiescence on HBV replication and indicated that the HBx-mediated exit from quiescence is critical for HBV replication in normal hepatocytes. While this study was consistent with previous reports which indicate that HBV replication is higher in G1 phase (Huang et al, 2004; Ozer et al, 1996), it is important to note that some studies indicate that HBV may replicate better in quiescent cells (Friedrich et al, 2005; Guidotti et al, 1997); however, in contrast to the described studies in cultured primary rat hepatocytes, the studies that suggested that HBV replicates better in quiescent cells did not distinguish between the G0 and G1 phase of the cell cycle. Studies in primary rat hepatocytes were also conducted to determine whether the HBx-mediated cell cycle arrest at the G1/S phase border was also required for HBV replication. When levels of HBV replication were measured in cultured primary rat hepatocytes that were transfected with siRNAs to decrease expression of p21, p27, or both p21 and p27, replication levels were considerably lower when both p21 and p27 levels were decreased, as compared to control samples or samples in which p21 or p27 levels were individually decreased (Gearhart & Bouchard, 2010b). Overall, the results of these studies showed that HBV replication in cultured primary rat hepatocytes is regulated by HBx-mediated G1 entry from G0 and cell cycle arrest at the G1/S phase border; HBV replication is inhibited when hepatocytes are in S phase.

Finally, to determine how HBx-mediated modulation of the cell cycle affects HBV replication, studies in cultured primary rat hepatocytes were conducted to define how cell cycle modulation influences the activity of the HBV reverse transcriptase/polymerase. The activity of the HBV polymerase was assessed in hepatocytes that were infected with

recombinant HBV-expressing adenoviruses and transfected with a p16 overexpression plasmid or siRNAs to p21 or p27 or both p21 and p27 (Gearhart & Bouchard, 2010b). The results of these studies demonstrated that entry of quiescent, primary rat hepatocytes into the G1 phase of the cell cycle is required for the activation of the HBV polymerase while progression into S phase did not affect the activity of the HBV polymerase (Gearhart & Bouchard, 2010b). In contrast, progression into S phase may inhibit HBV replication by causing a competition between the cell and the HBV replication machinery for available deoxynucleotide triphosphates (dNTPs). The levels of dNTPs in quiescent cells is low, and HBx modulation of the cell cycle is thought to increase the levels of cellular dNTPs that are available to the HBV polymerase [reviewed in (Yamashita & Emerman, 2006)]. Ribonucleotide reductase is the rate-limiting enzyme of DNA synthesis and is responsible for the synthesis of dNTPs [reviewed in (Elledge et al, 1992; Reichard, 1987)]. This enzyme contains two subunits, R1 and R2; R1 is expressed constitutively; however, the synthesis of R2, the catalytic subunit of ribonucleotide reductase, peaks closer to S phase, when the cells prepare for replication (Chabes & Thelander, 2000). Interestingly, HBx expression increased the levels of R2 in cultured primary rat and human hepatocytes, which would lead to the activation of ribonucleotide reductase and a possible increase in the number of available dNTPs (Gearhart & Bouchard, 2010b; Gearhart & Bouchard, 2011). Additional studies demonstrated that HBx elevation of R2 is required for HBV replication in cultured primary rat hepatocytes. Hydroxyurea, an inhibitor of ribonucleotide reductase (Thelander & Reichard, 1979), blocked HBV replication, as did transfection of a siRNA that targeted R2 (Gearhart & Bouchard, 2010b). Importantly, both hydroxyurea and the siRNA targeting R2 did not affect the activity of the HBV polymerase (Gearhart & Bouchard, 2010b). Cumulatively, the studies in primary hepatocytes demonstrated that HBx stimulates HBV replication by inducing hepatocytes to exit G0 and enter G1 to activate the HBV polymerase and increase the level of the R2 subunit of ribonucleotide reductase. Additionally, these studies confirmed that cultured rodent hepatocytes can serve as a powerful model system for studying HBx activities that are present in normal human hepatocytes, which are not always readily available for similar types of studies. Since deregulation of cell cycle checkpoints have been linked to cancer development [reviewed in (Collins et al, 1997; Ford & Pardee, 1999)], HBx modulation of cell cycle proliferation might also contribute to processes that influence the development of HBV-associated liver transformation by stimulating HBV replication and altering normal cell cycle control mechanisms in hepatocytes (Gearhart & Bouchard, 2010b). Overall, these observations indicate that HBx regulates the expression of cell cycle regulatory factors in normal hepatocytes when it is expressed in the absence of other HBV proteins and in the context of replication.

4.4.4 HBx and liver regeneration

Several studies have examined the effect of HBx expression on liver regeneration. In one study, moderate expression levels of HBx resulted in an inhibition of liver regeneration following a partial hepatectomy in HBx-transgenic mice (Tralhao et al, 2002). Overall inhibition of liver regeneration was also observed after a partial hepatectomy in mice that were transplanted with HBx-expressing liver cells (Tralhao et al, 2002). HBx-mediated inhibition of liver regeneration in HBx-transgenic mice occurred at the G1/S phase transition (Wu et al, 2006). HBx also caused an increase in the levels of alanine

aminotransferase and alpha-fetoprotein (AFP) that were detected after the partial hepatectomy, indicating that HBx expression made the hepatocytes more susceptible to damage during regeneration (Wu et al, 2006). HBx also caused abnormal apoptosis, fat accumulation, and impaired glycogen storage when HBx was expressed during periods of regeneration (Wu et al, 2006). In contrast, the results of one study suggested that HBx can increase cell cycle progression following a partial hepatectomy in HBx-transgenic mice; in this study the effect of HBx on regeneration was measured by BrdU incorporation, levels of PCNA, and reduced steady-state p21 protein levels (Hodgson et al, 2008). Finally, in another study in HBx-transgenic mice, hepatocytes in these mice had both increased proliferation capacity and increased apoptosis; whether increased hepatocyte proliferation was induced to compensate for the increased apoptosis, or whether increased apoptosis was in response to the increased hepatocyte proliferation was not directly addressed (Koike et al, 1998). Overall, these studies indicate that HBx can modulate liver regeneration; however, due to differences in the mouse strains and methods of analysis that were used to address the influence of HBx expression on liver regeneration, the exact impact of HBx on liver regeneration is not fully understood but could be a major contributor to the development of HBV-associated HCC.

4.5 HBx and calcium signaling

4.5.1 Calcium signaling overview

Calcium (Ca^{2+}) signaling controls a diverse range of cellular processes including cell proliferation, signal transduction, transcription, and apoptotic pathways [reviewed in (Berridge et al, 2003; Berridge et al, 2000; Bootman et al, 2001; Parekh, 2011)]. Calcium signals typically take the form of oscillating calcium spikes, and differences in the amplitude, frequency and spatial patterns of calcium oscillations will initiate different cellular responses, leading to a paradoxical combination of specificity and versatility in calcium signaling [reviewed in (Berridge et al, 2003; Berridge et al, 2000; Bootman et al, 2001; Parekh, 2011)]. The signaling functions of Ca^{2+} mainly depend on the calcium concentration in the cytosolic compartment. The cytosolic calcium level ($[Ca^{2+}]_c$) in most resting cells is maintained at around 100nM but can reach 1000nM upon stimulation [reviewed in (Berridge et al, 2000)]. The $[Ca^{2+}]_c$ is regulated by the dynamic interplay between calcium "ON" and "OFF" mechanisms that can increase or decrease $[Ca^{2+}]_c$ [reviewed in (Berridge et al, 2003; Berridge et al, 2000; Bootman et al, 2001)].

The calcium "ON" mechanism includes external calcium entry and internal calcium release from the endoplasmic reticulum (ER) [reviewed in (Berridge et al, 2003; Berridge et al, 2000; Bootman et al, 2001)]. A variety of plasma membrane calcium channels responsible for external calcium entry have been identified, including voltage-operated calcium (VOC) channels, receptor-operated calcium (ROC) channels, and store-operated calcium (SOC) channels [reviewed in (Berridge et al, 2003; Berridge et al, 2000; Bootman et al, 2001)]. The ER is the main internal calcium store, and inositol 1,4,5-triphosphate receptors (IP3R) and ryanodine receptors (RyR) are the best-characterized ER calcium-releasing channels [reviewed in (Berridge et al, 2003; Berridge et al, 2000; Bootman et al, 2001)]. In non-excitable cells, such as hepatocytes, cytosolic calcium signals mainly derive from ER calcium release. Extracellular agonists such as hormones or growth factors activate phospholipase C through

plasma membrane associated G-protein-coupled receptors or receptor tyrosine kinases, resulting in the breakdown of phosphatidylinositol 4,5-bisphoshate to produce inositol 1,4,5-triphosphate (IP3). IP3 binds to IP3Rs on the ER membrane and stimulates calcium release from the ER [reviewed in (Berridge, 1993; Berridge, 2009)]. IP3-linked ER calcium release is followed by extracellular calcium influx to replenish the ER calcium store, which is referred to as store-operated calcium entry (SOCE) (Putney, 1986). SOCE is essential for maintaining IP3-induced cytosolic calcium oscillations and normal ER functions, and also helps sustain elevated levels of $[Ca^{2+}]_c$ [reviewed in (Burdakov et al, 2005; Putney & Bird, 2008; Smyth et al, 2006)]. The process of SOCE is mediated by SOC channels, and the best characterized SOC channel is the calcium-release-activated calcium (CRAC) channel, which is highly selective for calcium and only activated by ER calcium depletion [reviewed in (Parekh, 2010)]. Recently, the ER transmembrane stromal interaction molecule (Stim) 1 (Stim1) and the plasma membrane proteins Orai family (Orai1, 2, and 3) have been identified as key molecular components of mammalian CRAC channels [reviewed in (Parekh, 2010)].

Once calcium signals are generated by the calcium "ON" mechanisms, they will be decoded and translated into different cellular processes by numerous calcium sensors. Some of these sensors, such as protein kinase C, bind to Ca^{2+} and are directly regulated in a Ca^{2+}-dependent manner. Other calcium sensors, such as calmodulin (CaM), act as intermediaries to couple calcium signals with distal targets and specific cellular responses. CaM is a versatile calcium sensor with two C-terminal and two N-terminal EF-hand Ca^{2+}-binding motifs; these motifs bind Ca^{2+} and induce conformational changes in CaM that facilitate activation of various downstream effectors, including CaM kinases (CaMKs) and calcineurin [reviewed in (Berridge et al, 2000; Parekh, 2011)]. Calcium signals also communicate with other signaling molecules including cyclic AMP, nitric oxide, phosphatidylinositol-3-OH (PI3) kinase, and MAPK [reviewed in (Berridge et al, 2000)]. Communication between calcium signaling and other signaling pathways greatly enhances the diverse functions of a calcium signal.

After a calcium signal has been executed, excess calcium will be rapidly removed from the cytosol by calcium pumps and exchangers; these pumps and exchangers constitute the cellular calcium "OFF" mechanisms [reviewed in (Berridge et al, 2000)]. The plasma membrane Ca^{2+}-ATPase pumps (PMCA) and Na+/Ca^{2+} exchangers can remove Ca^{2+} from the cytosol and transport Ca^{2+} to the extracellular environment. The sarco-endoplasmic reticulum ATPase (SERCA) pumps can pump Ca^{2+} from the cytosol into the ER (Berridge et al, 2003; Berridge et al, 2000; Bootman et al, 2001). Mitochondria also function as an important "OFF" mechanism by rapidly sequestering cytosolic Ca^{2+} during the rising phase of Ca^{2+} signals and then slowly releasing the Ca^{2+} back into the cytosol during the declining phase of Ca^{2+} signals (Berridge et al, 2000). Mitochondrial Ca^{2+} transport can buffer a harmful elevation of cytosolic Ca^{2+} and also increase the duration of Ca^{2+} signals in the cytosol. Mitochondria, therefore, have an important role in shaping both the amplitude and spatio-temporal patterns of Ca^{2+} signals [reviewed in (Berridge et al, 2000; Bootman et al, 2001)]. The major mitochondrial Ca^{2+} uptake mechanism is the mitochondrial Ca^{2+} uniporter (MCU) [reviewed in (Bernardi, 1999)]. Although the MCU has low Ca^{2+} affinity, a local high Ca^{2+} concentration in the ER-mitochondria microdomain can overcome this limitation and facilitate rapid mitochondrial Ca^{2+} uptake from the cytosol through the MCU during IP3-linked Ca^{2+} signaling (Rizzuto et al, 1993; Rizzuto et al, 1998). Mitochondrial Ca^{2+} efflux

mechanisms mainly include the mitochondrial Na+/Ca^{2+} exchanger and the MPTP; the MPTP is a mitochondrial protein complex that is thought to be composed of VDAC, the adenine nucleotide translocase (ANT) and cyclophilin D (CypD) [reviewed in (Bernardi, 1999)]. The MPTP has two conductance states: at the low conductance state, the MPTP can function as a mitochondrial Ca^{2+} efflux channel; at an irreversible high conductance state, mitochondria permeability transition will be induced and lead to the release of cytochrome C and the initiation of apoptosis (Ichas et al, 1997; Zoratti & Szabo, 1995).

4.5.2 Calcium signaling and the cell cycle

It is well recognized that Ca^{2+} signaling is involved at different stages of the cell cycle, especially during the early G1 phase as well as at the G1/S and G2/M transitions. Both extracellular Ca^{2+} and intracellular Ca^{2+} are required for cell proliferation. In one study, when the extracellular Ca^{2+} concentration was reduced from 1mM to 0.1mM, the rate of cell proliferation was gradually decreased (Hickie et al, 1983). The results of another study demonstrated that when intracellular Ca^{2+} stores were depleted with pharmacological agents, cell division was also blocked (Short et al, 1993). The role of Ca^{2+} signaling in progression through G1 and the G1/S boundary is particularly important. Ca^{2+} spikes have been observed in early G1 and near the G1/S boundary in cells [reviewed in (Kahl & Means, 2003)]. During the G1 phase, Ca^{2+} affects the expression of cyclin D1 by regulating the expression or activity of transcription factors such as fos, jun, and myc [reviewed in (Roderick & Cook, 2008)]; Ca^{2+} signals can also stimulate phosphorylation of Rb at the G1/S boundary (Takuwa et al, 1993).

CaM is a general Ca^{2+} sensor and relays Ca^{2+} signals to Ca^{2+}/CaM-dependent targets. The expression level of CaM is regulated during cell cycle progression, and a pronounced increase in CaM levels just before entering S phase has been observed in different cell types [reviewed in (Kahl & Means, 2003)]. Increased CaM levels accelerated the rate at which cells passed through the G1/S boundary, and a decrease in CaM levels prevented progression into S phase, indicating that CaM can control the rate of entering into S phase (Rasmussen & Means, 1989). The addition of CaM inhibitors early in the G1 phase could completely inhibit DNA synthesis, but CaM inhibitors did not affect DNA synthesis if the inhibitors were added later in G1 after Rb hyperphosphorylation, suggesting that the requirement for Ca^{2+}/CaM in late G1 phase is before Rb hyperphosphorylation (Takuwa et al, 1992; Takuwa et al, 1993). Although the detailed molecular mechanisms that underlie these Ca^{2+}/CaM effects remain unknown, it is evident that Ca^{2+} signaling can affect multiple signaling pathways to regulate G1 progression.

Two Ca^{2+}/CaM-dependent targets, calcineurin and CaMKs, have been shown to play an important role in cell-cycle progression and cell proliferation [reviewed in (Kahl & Means, 2003)]. The multifunctional CaMKs are a family of serine/threonine protein kinases that includes CaMKI, CaMKII, and CaMKIV. The auto-inhibition of CaMKs is removed upon the binding of Ca2+/CaM, which stimulates CaMK kinase activity [reviewed in (Kahl & Means, 2003)]. CaMKs are also associated with different aspects of cell cycle progression. In both normal and transformed cells, inhibition of CaMKs prevented G1 progression, suggesting that CaMKs are required for G1 progression [reviewed in (Kahl & Means, 2003)]. *In vitro*, CaMKI and CaMKII phosphorylated and increased the activity of cdc25C, a phosphatase responsible for cdc2 activation at the G2/M transition (Patel et al, 1999). Calcineurin is a

Ca^{2+}/CaM-dependent phosphatase, and its enzyme activity is stimulated by binding of Ca^{2+}/CaM to its regulatory domain [reviewed in (Kahl & Means, 2003)]. Calcineurin can affect cyclin D1 expression and control G1 progression by regulating cAMP-responsive element binding protein (CREB), which binds to the cyclin D1 promoter (Schneider et al, 2002). NFAT activation by calcineurin can also induce the expression of c-myc and increase the expression of E2F and cyclin E [reviewed in (Roderick & Cook, 2008)]. Finally, inhibition of calcineurin suppresses CDK2 activity because of increased expression of p21 or reduced cyclin E levels (Khanna & Hosenpud, 1999; Tomono et al, 1998).

Because of the pivotal role of calcium signaling in cell proliferation, abnormal Ca^{2+} signaling may contribute to the development of cancer [reviewed in (Parkash & Asotra, 2010; Roderick & Cook, 2008)]. The involvement of Ca^{2+}, CaM, calcineurin, and NFAT signaling in carcinogenesis has been implicated in many different human malignant tumors. NFAT-mediated signaling can affect the development of cancer by upregulation of VEGF to stimulate angiogenesis, by upregulation of c-Myc to prompt tumor cell proliferation, and by upregulation of Cox-2 to facilitate tumor cell migration (Buchholz & Ellenrieder, 2007). Ca^{2+} signaling can also regulate oncogenic signaling pathways by contributing to the magnitude and duration of Ras and ERK activation [reviewed in (Roderick & Cook, 2008)]. Furthermore, deregulation of Ca^{2+} signals that are involved in centrosome replication and separation can result in aberrant mitotic spindles and genetic instability, contributing to the development of cancer [reviewed in (Roderick & Cook, 2008)]. Taken together, remodeling of Ca^{2+} signaling in cell proliferation may act as an oncogenic mechanism facilitating the development of cancer.

4.5.3 HBx modulation of the cell cycle requires calcium signaling

Various studies suggest that HBx modulates cytosolic Ca^{2+} signaling, which likely acts as an initiator of other reported HBx activities (Bouchard et al, 2006; Bouchard et al, 2001b; Gearhart & Bouchard, 2010a; Gearhart & Bouchard, 2010b). This notion is supported by studies of regulation of cell cycle and cell proliferation in cultured primary rat hepatocytes (Gearhart & Bouchard, 2010a; Gearhart & Bouchard, 2010b). Studies in cultured primary rat hepatocytes have demonstrated that HBx induced quiescent hepatocytes to enter the G1 phase of cell cycle but not to proceed to S phase and that HBV replication was reduced when cultured primary rat hepatocytes entry into G1 phase was blocked or when cultured primary rat hepatocytes entered S phase (Gearhart & Bouchard, 2010a; Gearhart & Bouchard, 2010b). Of significance to the role of HBx modulation of Ca^{2+} signals, these studies in cultured primary rat hepatocytes showed that treatment with BAPTA-AM, an intracellular Ca^{2+} chelator, inhibited HBx modulation of cell cycle regulatory proteins and abolished HBV replication, suggesting that HBx regulation of cell proliferation to stimulate HBV replication requires Ca^{2+} signaling (Gearhart & Bouchard, 2010a; Gearhart & Bouchard, 2010b). This conclusion is consistent with previous studies in HepG2 cells that showed that the modulation of Ca^{2+} signaling by HBx is essential to stimulate HBV replication. In HepG2 cells, the replication of HBx-deficient HBV was significantly reduced and was rescued by co-transfecting with an HBx-expressing plasmid or by simply increasing cytosolic Ca^{2+} levels; BAPTA-AM treatment inhibited HBV replication (Bouchard et al, 2001b). Furthermore, the activation of Pyk2/FAK and Src kinase pathway has been identified as one of the downstream pathways of HBx-regulated Ca^{2+} signals that stimulates HBV replication in HepG2 cells. HBx activation of Pyk2/FAK and Src kinases

was Ca^{2+}-dependent, and the inhibition of Pyk2/FAK and Src kinases blocked HBx-stimulated HBV replication in HepG2 cells (Bouchard et al, 2003; Bouchard et al, 2006; Bouchard et al, 2001b). Taken together, these studies suggest that HBx modulation of Ca^{2+} signaling can stimulate HBV replication in both HepG2 cells and cultured primary rat hepatocytes. In addition, these studies show that HBx regulation of hepatocyte proliferation is directly linked to HBx stimulation of Ca^{2+} signals.

An unanswered question is precisely how HBx modulates cellular Ca^{2+} signals. The results of many studies suggest that HBx affects Ca^{2+} signaling by regulating the MPTP. Because cyclosporin A (CsA) inhibits both the MPTP and calcineurin and FK506 is only a calcineurin inhibitor that does not affect MPTP, the combined use of these two inhibitors has helped to define whether MPTP is involved in a specific cellular process. Importantly, CsA treatment, but not FK506 exposure, blocked the effect of HBx on cell cycle regulatory proteins such as p15, p16, p21, and p27 and HBx stimulation of HBV replication in cultured primary rat hepatocytes, indicating that HBx modulation of cell cycle factors in cultured primary rat hepatocytes is MPTP-dependent (Gearhart & Bouchard, 2010b). Similar results were observed in HepG2 cells that were treated with CsA; CsA treatment blocked HBx stimulation of HBV replication and activation of Pyk2 and FAK (Bouchard et al, 2006; Bouchard et al, 2001b). Finally, the direct link between HBx modulation of Ca^{2+} signaling

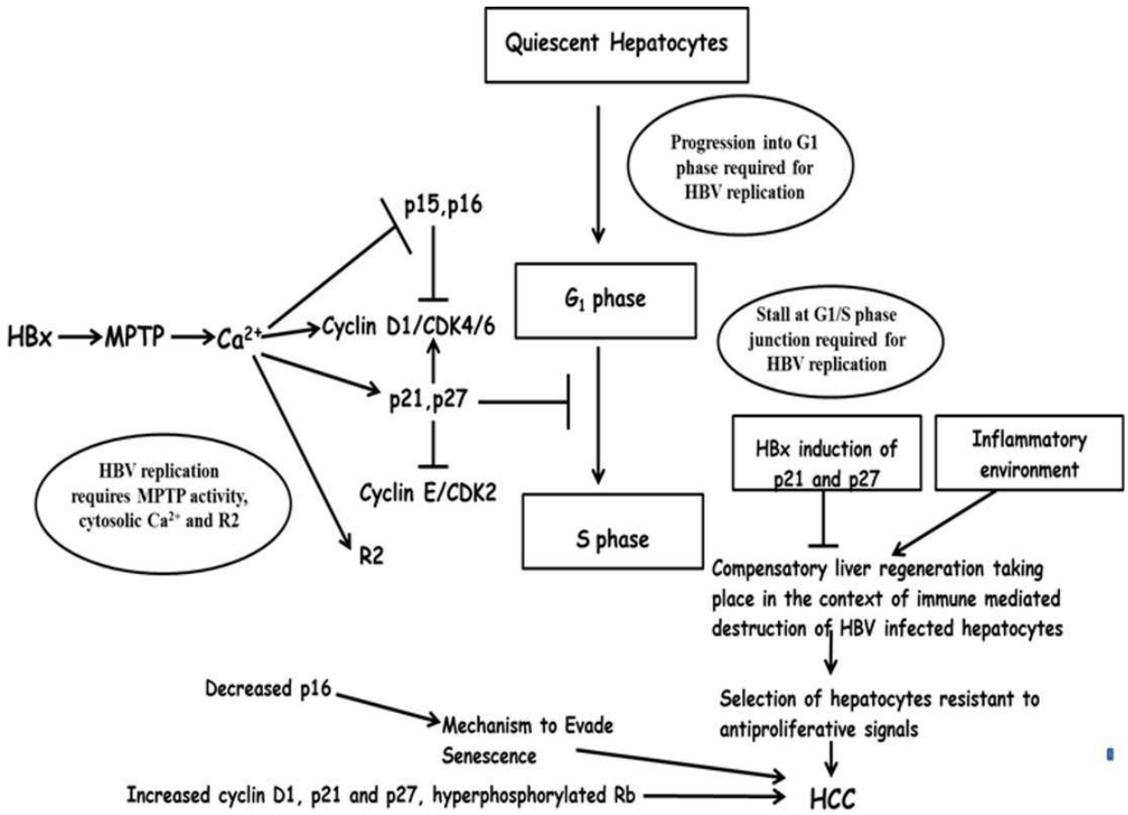

Fig. 4. HBx modulation of cell cycle in primary hepatocytes may lead to the development of HCC. Refer to Figure 2 for the other activities of HBx that could play a potential role in the development of HBV-induced HCC. See text and references for details.

and the MPTP was established in HepG2 cells. In these cells, HBx, both by itself and in the context of HBV replication, increased the basal cytosolic Ca^{2+} level; this increase was blocked by CsA treatment, suggesting that MPTP is involved in the regulation of cytosolic calcium signals (McClain et al, 2007). Cumulatively, these studies suggest that HBx affects cellular Ca^{2+} signaling in a manner that is dependent on the MPTP. Whether CaM acts a mediator of HBx-induced calcium signals remains to be investigated. Remodeling of Ca^{2+} signals and the impact of this on cell proliferation could contribute to carcinogenesis, and HBx modulation of cellular Ca^{2+} signals may contribute to the development of HBV-associated HCC. The precise mechanism used by HBx to regulate cellular Ca^{2+} signals through the MPTP remains unclear, and a better understanding of this HBx activity may provide new targets for treatment of chronic HBV infections and prevention of the development of HBV-associated HCC.

4.6 Conclusions and future directions

4.6.1 HBx modulation of calcium signaling and the cell cycle in hepatocarcinogenesis

HBx can influence a number of cellular activities that could alter normal hepatocyte physiology and predispose hepatocytes to transformation; these activities may include HBx regulation of cellular signal transduction, transcription, proliferation, and apoptotic pathways, as well as the direct interaction of HBx with cellular proteins such as UV-DDB and p53. In this chapter, we have focused on HBx modulation of hepatocyte proliferation pathways and cellular calcium signaling as HBx activities that could be major contributing factors to the development of HBV-associated HCC (Figure 4). Deregulation of calcium signaling can alter normal cellular physiology, thus potentially contributing to cancer development [reviewed in (Parkash & Asotra, 2010)]. As the upstream mediator of many HBx effects (Bouchard et al, 2006; Bouchard et al, 2001b; Gearhart & Bouchard, 2010a; Gearhart & Bouchard, 2010b), alterations in calcium signaling could play a major role in HBV-induced HCC. Many alterations of cell cycle regulatory proteins that have been identified in HCC are similar to alterations in cell cycle pathways that have been observed in primary hepatocytes when HBx is expressed in the absence of other proteins and in the context of HBV replication (Biden et al, 1997; Burkhart & Sage, 2008; Csepregi et al, 2010; Deane et al, 2001; Gearhart & Bouchard, 2010a). There are numerous ways in which HBx regulation of hepatocyte proliferation pathways could influence HCC development. For example, down-regulation of p16 by HBx enabled hepatocytes to evade senescence, a potent tumor suppressive mechanism (Kim et al, 2010). Overexpression of cyclin D1 promotes hepatocarcinogenesis (Deane et al, 2001), and HBx-mediated upregulation of cyclin D1 could affect the development of liver cancer. Stimulation of HBV replication by modulating the cell cycle could, in itself, generate a more inflammatory environment due to higher levels of HBV replication; increased inflammation could enhance the oncogenic environment in the liver. Overall, the HBx-mediated induction of cell cycle entry of quiescent primary hepatocytes during the course of a chronic HBV infection could alter the physiology of hepatocytes to favor carcinogenesis, and signal transduction pathways activated by HBx, including pathways involved in cell proliferation and survival, have been implicated in hepatocarcinogenesis [(Chen et al, 2010; Chung et al, 2004; Hsieh et al, 2011); reviewed in (Ierardi et al, 2010; Rodrigues & Barry, 2011; Thompson & Monga, 2007; Torbenson et al, 2004; Whittaker et al, 2010)]. In addition, in the context of an inflammatory environment where HBV-infected hepatocytes are being destroyed by inflammatory

responses, any mechanisms that inhibit regeneration, such as HBx induction of p21 or p27, might eventually lead to compensatory mechanisms that select for cells that have become resistant to anti-proliferative signals. Repeated cycles of forced hepatocyte regeneration occurring in the context of the inflammatory environment of an HBV-infected liver could eventually contribute to HCC development.

4.6.2 Future directions

While recent studies are beginning to define the activities of HBx in normal hepatocytes, in the livers of small animal models, and in the context of HBV replication, the precise molecular mechanisms that underlie many HBx activities remain unknown and should be the focus of future studies. Although a bewildering array of functions have been linked to HBx expression, these activities may simply reflect the cell-specific consequence of a limited number of initiating events that are controlled by a small number of primary HBx activities. Due to the paucity of available model systems for studying direct HBV infections and the low level of HBx expression during HBV replication, identifying HBx activities during HBV replication in authentic normal human hepatocytes will likely remain a challenge. Studies in other model systems such as cultured primary rodent hepatocytes will provide valuable models for characterizing HBx activities in normal hepatocytes. The major focus of this chapter has been HBx regulation of calcium signaling and cell proliferation pathways and how these HBx activities regulated HBV replication and may influence the development of HCC; consequently, in the following section we will focus on potential future directions that are relevant to these HBx activities.

The role of the Pyk2/FAK-Src-Ras-Raf-MAPK pathway in HBx regulation of hepatocyte proliferation pathways remains unexplored and an important future area of investigation. HBx stimulation of the Pyk2/FAK-Src-Ras-Raf-MAPK pathway could profoundly affect hepatocyte physiology and modulate carcinogenic processes. Results from several studies have suggested that expression levels of p21, p27, and cyclin D1 can be regulated by MAPK signaling [(Bottazzi et al, 1999; Liu et al, 1996); reviewed in (Kerkhoff & Rapp, 1998)]. Importantly, the results of one study in mouse hepatocytes identified a role of prolonged MAPK signaling in HBx-induced elevation of p21 and p27 (Qiao et al, 2001). Whether the Pyk2/FAK-Src-Ras-Raf-MAPK pathway, which has been linked to HBx elevation of calcium, is directly responsible for HBx regulation of hepatocyte proliferation pathways and HBV replication in primary hepatocytes awaits further investigation. Additionally, the mechanism that underlies HBx regulation of cyclin D1 in normal hepatocytes is unknown. The cyclin D1 promoter contains several binding sites for the transcription factor NF-κB (Hinz et al, 1999), and studies have shown that HBx can activate NF-κB in cultured primary rat hepatocytes (Clippinger et al, 2009). One study in Chang liver cells suggested that the HBx-mediated increase in cyclin D1 expression was caused by HBx activation of NF-κB (Park et al, 2006); however, whether HBx upregulates expression of cyclin D1 through activation of NF-κB in a normal hepatocyte has yet to be determined and should be the subject of future studies. Activation of the Wnt pathway, inactivation of GSK3-β, and stabilization of β-catenin have also been linked to activation of the cyclin D1 transcription promoter [reviewed in (Monga, 2011)], and it was recently shown that HBx can activate the Wnt pathway (Cha et al, 2004; Hsieh et al, 2011), increase the phosphorylation and inactivation of GSK3-β (Cha et al, 2004; Yang et al, 2009), and increase β-catenin

accumulation in some HCC cell lines (Cha et al, 2004; Hsieh et al, 2011). Whether HBx regulates cyclin D1 expression through activation of the Wnt signaling pathway in normal hepatocytes and in the context of HBV replication is unknown; inactive GSK3-β and increased β-catenin accumulation are associated with HCC development (Ban et al, 2003). An important HBx activity that could also affect cyclin D1 levels and requires further investigation is HBx stimulation of DNMT1 expression and the resulting elevation of methylation of the p16 promoter and decreased p16 expression; this HBx activity was linked to elevation of cyclin D1 and phosphorylation of Rb in HepG2 cells (Jung et al, 2007; Zhu et al, 2007). Although HBx regulation of p16 levels in cultured primary rat and human hepatocytes was linked to calcium signaling (Gearhart & Bouchard, 2010a), the possible role for DNMT1 in decreased expression of p16 was not assessed in these studies and could have important implications for HCC development.

The interaction of p53 and HBx, whether direct or indirect, remains a subject of considerable debate, and a comprehensive analysis of precisely how HBx or p53 each affects activities of the other protein could provide important insights into mechanisms that link HBV infections and HBx expression to HCC development. Although p53 stimulation of p21 expression has been extensively characterized (el-Deiry et al, 1993), whether p53 affects HBx regulation of p21 and hepatocyte proliferation pathways in normal hepatocytes and in the context of HBV replication remains to be determined. Interestingly, HBx was shown to induce p21 expression in a both a p53-dependent and independent manner (Ahn et al, 2002; Park et al, 2000). An important focus of future studies should be a comprehensive analysis of the interplay between HBx and p53 activities in normal hepatocytes and how this influences HBV replication and the development of HCC-associated HCC.

Although the future studies described above would address the impact of HBx expression on hepatocyte physiology, an equally important focus for future studies is to determine precisely how HBx modulation of the cell cycle directly affects HBV replication. Studies in primary rat hepatocytes have clearly shown that hepatocyte exit from G0 and subsequent arrest in G1 is required for HBV replication and for activation of the HBV polymerase (Gearhart & Bouchard, 2010a; Gearhart & Bouchard, 2010b); however, exactly how this HBx activity stimulates the polymerase remains unknown. Moreover, effects of cell cycle modulation on other HBV proteins that are required for HBV replication have not been investigated. The HBV core protein is phosphorylated on three serine residues; differential phosphorylation of these serines is required for various stages of HBV replication (Melegari et al, 2005). Whether HBx regulation of the cell cycle affects core phosphorylation in normal hepatocytes and whether this modulates HBV replication remains to be explored. The results of several studies have identified putative candidate kinases that are responsible for these phosphorylation events (Daub et al, 2002; Duclos-Vallee et al, 1998; Kann & Gerlich, 1994; Yeh et al, 1993); however, the events and kinase(s) that result in the phosphorylation of HBV core in hepatocytes are generally not well understood. It is possible that the kinase responsible for phosphorylation of the HBV core protein is activated by the HBx regulation of hepatocyte proliferation pathways, and specifically identifying this kinase(s) in HBV-infected hepatocytes will be an important focus of future studies. Finally, the upregulation of the R2 subunit of ribonucleotide reductase by HBx, and the dependence of HBV replication on upregulation of R2 expression suggest that HBx may cause cell cycle entry in order to increase the availability of dNTPs. Recently, it was shown that HBV can increase the cellular concentration of dNTPs in HepG2.2.15 cells, as compared to HepG2 cells (Cohen

et al, 2010). Although these findings are intriguing, this increased pool of dNTPs was not directly linked to HBx and not confirmed in HBV-infected normal hepatocytes. Consequently, an important subject of future studies should be to determine whether an increase in dNTP concentration is the ultimate goal of the HBx-mediated cell cycle modulation and whether dNTPs are a limiting factor during HBV replication in hepatocytes. Ribonucleotide reductase could be a new drug target to inhibit HBV replication in HBV-infected individuals; recent studies have shown that inhibition of the R2 subunit of ribonucleotide reductase can reduce the growth potential of cancer cells (Heidel et al, 2007). Overall, the results of future studies that address HBx activities in authentic hepatocytes and in the context of HBV replication should focus on identifying primary HBx activities, thereby generating potential new therapeutic targets to inhibit HBV replication and the development of HBV-associated HCC.

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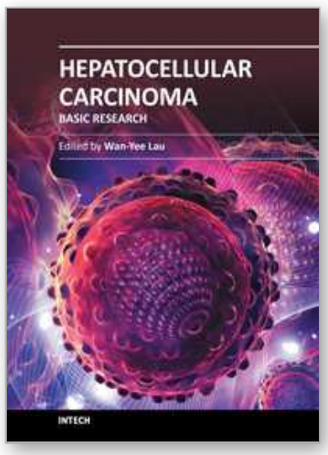

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Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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