

## HEPATITIS B VIRUS X ANTIGEN (HBxAg) AND CELL CYCLE CONTROL IN CHRONIC INFECTION AND HEPATOCARCINOGENESIS

Mark A. Feitelson<sup>1,2</sup> Helena M. G. P. V. Reis,<sup>1</sup> Jie Liu,<sup>1</sup> Zhaorui Lian<sup>1</sup> and Jingbo Pan<sup>1</sup>

<sup>1</sup> Department of Pathology, Anatomy and Cell Biology, <sup>2</sup> Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107

### TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Dysregulation of apoptosis and cell cycle control in hepatocarcinogenesis
4. Genetic contribution of HBV and related viruses to hepatocarcinogenesis
5. HBxAg promotes cell cycle progression and growth
  - 5.1. HBxAg promotes growth and tumorigenesis by accelerated transit through cell cycle checkpoints
  - 5.2. Consequences of cell cycle progression on virus gene expression/replication and state of hepatocellular differentiation
  - 5.3. HBxAg promotes cell cycle progression by blocking negative growth regulatory pathways
  - 5.4. activation in promoting cell cycle progression in tumor development
  - 5.5. HBxAg promotes cell cycle progression by modulating apoptotic signaling
  - 5.6. HBxAg binding partners and cell cycle progression
  - 5.7. HBxAg activation of signaling pathways involved in cell cycle progression may be related to state of cellular differentiation
  - 5.8. HBxAg triggered genetic instability may promote cell cycle progression
  - 5.9. HBxAg and oncogene activation in cell cycle progression
6. Summary and future prospects
7. Acknowledgements
8. References

### 1. ABSTRACT

Hepatitis B and related viruses that infect mammalian hosts encode the “X” protein that has been shown to contribute importantly to the pathogenesis of chronic liver disease (CLD) and to the development of hepatocellular carcinoma (HCC). In a variety of tissue culture systems, hepatitis B virus (HBV) X antigen, or HBxAg, has been shown to trigger apoptosis, while other evidence suggests that HBxAg inhibits apoptosis and stimulates the cell cycle by constitutively activating a number of signaling pathways that are important for hepatocellular growth and survival. These apparently contrasting properties of HBxAg may be associated with differences in the X protein itself, since carboxy-terminal truncated forms of HBxAg appear to be associated with HCC lesions. Alternatively, or in addition, these differences may be due to the cell type, state of cell differentiation, and whether expression occurs in resting or dividing cells. Further, the association between HBxAg expression and chromosomal instability, may also contribute to the apparently contrasting fates of HBxAg positive cells. It is proposed that in many of these systems, the different outcomes of HBxAg expression may be due to the nature of the cellular response to HBxAg, and not due to differences in the fundamental properties of HBxAg, the latter of which promote cell survival, cell cycle progression, and the development of HCC.

### 2. INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most prevalent tumor types worldwide, with an estimated more than 250,000 new cases diagnosed each year (1).

There are many etiological factors that contribute to the development of HCC, including chronic infection with hepatitis B virus (HBV) and/or hepatitis C virus (HCV), chronic alcoholism, hormones, dietary factors (e.g., aflatoxin B<sub>1</sub> in contaminated peanuts), and a variety of genetic predispositions (e.g., hereditary tyrosinemia, hemochromatosis, and alpha-1 antitrypsin deficiency) (2, 3). Among people who are chronically infected with hepatitis B or C, 350 million HBV carriers (4) and 170 million HCV infected patients are at high risk for tumor development (5). In particular, studies in the early 1980's showed that the HBV carrier state and associated chronic liver diseases (CLD; e.g., hepatitis and cirrhosis) are major risk factors for the development of HCC (6, 7). The relative risk of HBV carriers developing HCC is 200:1, which is one of the tightest relationships between an environmental carcinogen and a tumor type (6, 7). However, the molecular basis for HBV associated HCC has not been elucidated.

The incidence of HCC is geographically variable, with the highest frequencies observed in subSaharan Africa and in the Far East among countries where HBV is endemic. HCC is also predominantly male associated, with male:female ratios of up to 2:1 (8). HCC is rapidly fatal, with a life expectancy of about 6 months from time of diagnosis, and a < less than 3% survival rate for untreated cancer over 5 years. Death is often due to liver failure associated with cirrhosis and/or rapid tumor growth. Although early HCC may be cured by surgical resection and/or localized therapies at the tumor site, the fact that many tumors are asymptomatic, means that most patients at risk will not be diagnosed in time. These patients often

## HBxAg and cell cycle progression

present with inoperable HCC, which usually reflects the presence of large and/or multiple HCC nodules. Despite the high incidence and mortality, the development of an effective vaccine for HBV, combined with its universal administration to newborns in endemic countries, will significantly lower the incidence of HCC within the next generation. There is also evidence that antiviral treatment with interferon (9) may lower the risk for HCC development. Despite these advances, the mechanism(s) whereby chronic HBV infection results in HCC, is still a matter of considerable debate.

Clearly, controls on cell cycle progression are missing in tumor compared to normal cells. However, it is not known whether altered cell cycle control is the cause or consequence of tumorigenesis. In addition, the events that trigger alterations in cell cycle control in hepatocarcinogenesis are not clear, although there is increasing evidence that HBV and HCV encode proteins that act by deregulating the control of both the cellular apoptotic responses to inappropriate growth stimulatory signals and the ability of cells to regulate their own cell cycle machinery. Hence, this review focuses on how the HBV encoded X antigen, or HBxAg, stimulates the cell cycle in liver cells. This appears to be done by blocking normal cellular apoptotic responses, by constitutively activating selected signaling pathways that override apoptosis, and by directly stimulating entry of cells into the cell cycle.

### 3. DYSREGULATION OF APOPTOSIS AND CELL CYCLE CONTROL IN HEPATOCARCINOGENESIS

A fundamental feature in the pathogenesis of cancer is deregulation of cell growth control, which involves alterations in cellular proliferation and apoptosis. Dysregulation in proliferation and apoptosis has also been identified as being of central importance to the development of HCC (10, 11). In the context of CLD, HBV (and HCV) infected cells are targeted for damage and destruction by cell mediated immune responses (12), which result in the removal of virus infected hepatocytes and the regeneration of surviving hepatocytes to help restore a fully functional liver, which is important for maintaining homeostasis. Immune mediated removal of virus-infected hepatocytes during multiple bouts of hepatitis commonly results in the development of fibrosis, or scarring, which progressively adds acellular extracellular matrix (mostly connective tissue) to the liver in place of hepatocytes (Figure 1). In time, the strands of connective tissue link up with each other in three dimensions, resulting in cirrhosis. In normal, uninfected liver, hepatocytes are nonreplicating, arranged in sheets, and are well vascularized, with one surface of the hepatocyte in direct contact with the bloodstream (13). In a cirrhotic liver, however, the hepatocytes are often proliferating spheres of cells within fibrous capsules that are poorly vascularized. The direct relationship between the hepatocytes and the bloodstream is lost, with basement membranes appearing during the development of cirrhosis. This environment is hypoxic and is often deficient of serum-based growth factors, suggesting that adaptive (preneoplastic?) changes occur in cirrhotic

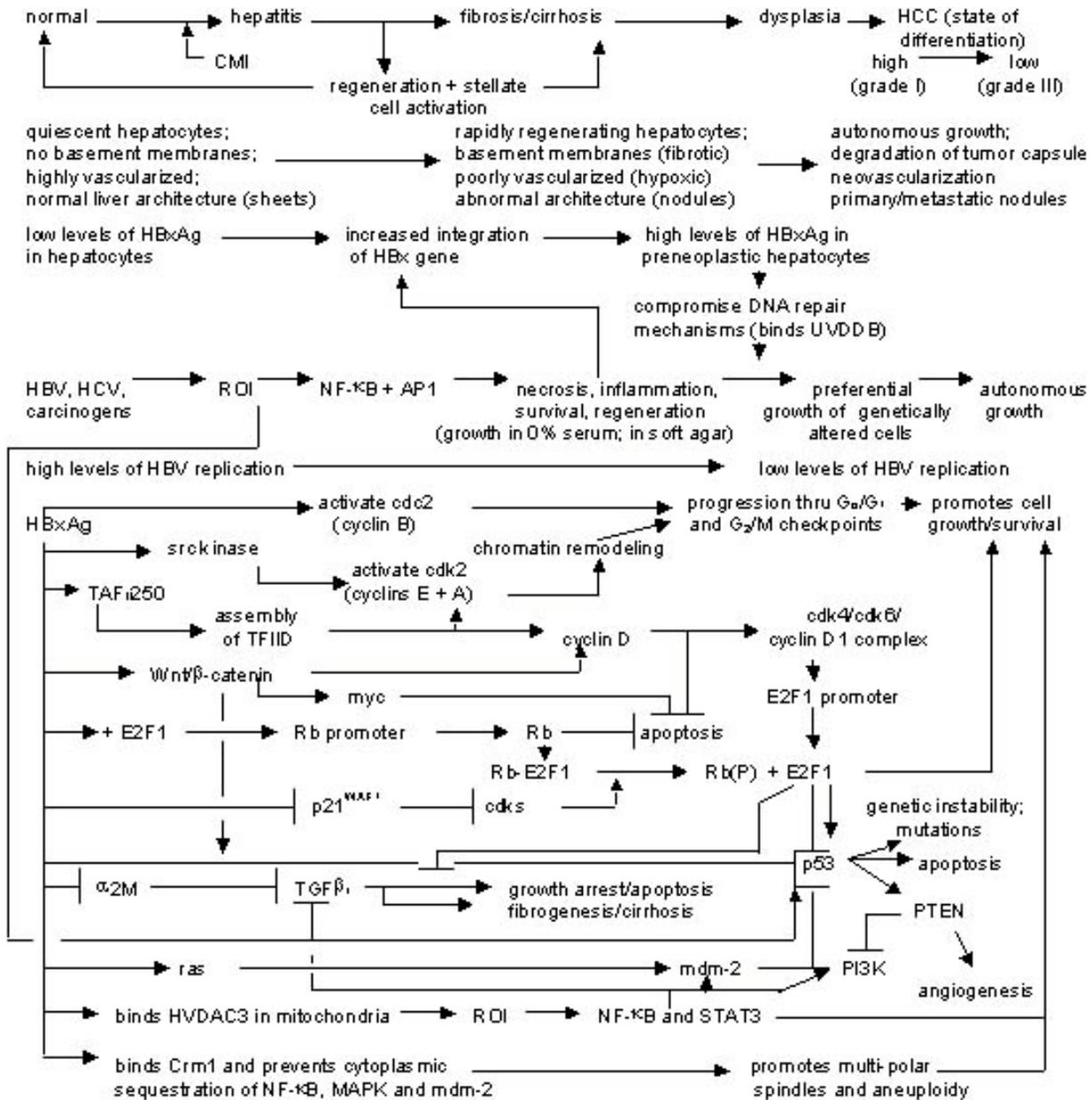
nodules that promote growth factor independent growth and survival. The overall impact is a change from quiescent, nonreplicating hepatocytes in a liver with normal architecture to rapidly replicating hepatocytes in a cirrhotic liver, demonstrating that the pathogenesis of chronic HBV infection is associated with cell cycle progression in a tissue type which otherwise is known for very little cellular turnover (Figure 1).

In the pathogenesis of CLD, the production of reactive oxygen intermediates (ROI) stimulate the activities of oxygen sensitive signaling factors, such as nuclear factor kappa B (NF-kappaB) and activating protein-1 (AP-1), which are hepatoprotective (14-16), in that they help to prevent the destruction of hepatocytes during periods of chronic hepatitis. They may also help to promote the growth and survival of hepatocytes under hypoxic conditions (Figure 1). This has not only been implied in the clinical literature, but also from hepatitis B surface antigen (HBsAg) transgenic mice that overproduce HBsAg to toxic levels, resulting in the destruction of hepatocytes, followed by hepatocellular proliferation and cell-mediated immune responses that generate considerable levels of intrahepatic ROI (17, 18). These mice eventually develop HCC (19). Although the overproduction of HBsAg is unrelated to the pathogenesis of HBV associated HCC in people, it does support the hypothesis that the pathogenesis of HCC is immune mediated. This was formally shown in experiments whereby the adoptive transfer of HBsAg specific cytotoxic T lymphocytes (CTL) into thymectomized, irradiated and bone marrow reconstituted HBsAg overproducing transgenic mice resulted in the appearance of chronic hepatitis, followed by hepatocellular turnover, and the appearance of HCC (20). This model is compatible with the observations that HBV carriers without liver disease have a low risk for the development of HCC, while carriers with CLD are at very high risk (6). Although HCC nodules have been shown to arise from cirrhotic/dysplastic nodules, cirrhosis is not required for the development of HCC, since there are many HBV carriers who develop HCC on a background of hepatitis. Moreover, woodchucks chronically infected with the HBV-related woodchuck hepatitis virus (WHV) do not develop cirrhosis, but develop HCC on a background of chronic hepatitis (21). Ground squirrels infected with the related ground squirrel hepatitis virus (GSHV) also develop CLD and HCC, although the chronic hepatitis is usually milder than in woodchucks, and the frequency of HCC is correspondingly lower (22, 23). A common denominator in all of this is hepatocellular destruction and rapid regeneration in the presence of ROI, suggesting that deregulation of apoptosis and loss of cell cycle control are central features in the development of cancer with HBV and related viruses.

### 4. GENETIC CONTRIBUTION OF HBV AND RELATED VIRUSES TO HEPATOCARCINOGENESIS

In natural infections, HCC developed in about 30% of GSHV infected ground squirrels 4-5 years of age (22) compared with nearly 100% of WHV infected woodchucks 2-3 years of age (24, 25). Infection of

## HBxAg and cell cycle progression



**Figure 1.** Biochemical pathways altered by HBxAg in the pathogenesis of HCC with emphasis on how these pathways alter the cell cycle machinery. Please see the text for additional details.

woodchucks with GSHV (26) resulted in only about 40% of the animals developing HCC at 4-5 years of age (27). The differences in the behavior of these two viruses in a single host suggest that the differences in tumorigenicity are in the viruses themselves, and that these viruses make a genetic contribution to cancer. The latter is further strengthened by the finding that WHV is a complete carcinogen (25). Interestingly, a common feature of chronic infection is that fragments of HBV DNA become integrated into many sites within multiple chromosomes (28, 29). Close examination of the integrated viral DNA sequences

show that they often span the HBx gene, and sometimes, the envelope gene (28, 30-32). Integrated HBV DNA often produce X mRNA (34, 35), HBxAg protein (35, 36) (and sometimes truncated envelope polypeptides) which are active in *trans*-activation assays (37-40). HBxAg is the most prevalent virus antigen in the liver and tumors of HBV carriers with CLD, and strongly correlates with CLD in human and woodchuck infections (33-36, 41, 42), suggesting that it promotes the persistence of the carrier state by altering the growth and survival of infected hepatocytes during infection. As detailed below, the

## HBxAg and cell cycle progression

persistent production and function of HBxAg now appears essential for hepatocellular transformation, and likely represents the major genetic contribution of these viruses to the development of HCC.

### 5. HBxAg PROMOTES CELL CYCLE PROGRESSION AND GROWTH

#### 5.1. HBxAg promotes growth and tumorigenesis by accelerated transit through cell cycle checkpoints

There is considerable evidence that HBxAg promotes cell growth and tumorigenesis. For example, HBxAg promotes the growth of the human hepatoblastoma cell line, HepG2, in complete medium as well as in serum free medium, promotes anchorage independent growth of HepG2 cells in soft agar, and significantly accelerates the development of subcutaneous tumors in nude mice (43-46). HBxAg also transforms NIH3T3 cells, which was accompanied by elevated *c-myc* expression (47). HBxAg also transformed the immortalized but nontumorigenic mouse hepatocyte cell line, FMH-202 (48, 49). HBx transgenic mice with sustained, high levels of HBxAg developed HCC (50, 51), while HBx transgenic mice with little or undetectable X expression did not (52), suggesting a dose dependent cause and effect. In addition, integrated HBV DNA fragments from human HCCs have been shown to transform FMH-202 cells (53), while integrated WHV and adjacent *c-myc* sequences from woodchuck HCCs have been shown to give rise to HCC in transgenic mice (54). These findings not only underscore the contribution of HBxAg to hepatocarcinogenesis, but also suggest that HBxAg may do this, in part, by stimulating cell cycle progression.

In this context, HBxAg has been shown to stimulate cellular DNA synthesis in several cell types (25, 47). In fibroblasts, for example, HBxAg stimulated progression through the G<sub>0</sub>/G<sub>1</sub> checkpoint, effectively moving quiescent cells into S phase (55). Independent observations have shown that HBxAg not only stimulates the emergence of liver cells from quiescence, but also accelerates their transit through the G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M checkpoints (56) (Figure 1). This is associated with the activation of *cdk2* and *cdc2*, which in turn form active associations with cyclins E and A or cyclin B, respectively (56). HBxAg cooperates with the TATA-binding protein-associated factor 250 (TAF<sub>II</sub>250), a transcriptional coactivator, for activation of the cyclin A promoter and for early cell cycle transit from G<sub>0</sub> to G<sub>1</sub> (57). TAF<sub>II</sub>250 is essential for the assembly of TFIID complexes, and triggers chromatin remodeling through an intrinsic histone acetyltransferase activity that results in the activation of cyclin A and cyclin D genes, both of which are required for cycle progression (58) (Figure 1). The subsequent formation of cyclin A-cdk2 complexes, and deregulation of early cell cycle checkpoints, was also dependent upon the ability of HBxAg to stimulate cytoplasmic src kinase signaling (57), indicating that the activation of both TAF<sub>II</sub>250 and src signaling may contribute importantly to how HBxAg promotes the entry of quiescent cells into the cell cycle.

#### 5.2. Consequences of cell cycle progression on virus gene expression/replication and state of hepatocellular differentiation

The ability of HBxAg to promote entry of cells into the cell cycle have profound effects upon the ability of HBV to replicate. In tissue culture cells replicating HBV, there is an inverse relationship between HBV gene expression/replication and the state of cellular growth, with the highest levels of HBV being made in confluent cultures and relatively little made during log phase growth (59-61). In the context of CLD, low levels of HBV gene expression and replication in regenerating hepatocytes would partially shield them against immune mediated destruction until regeneration nears completion. This may contribute to virus persistence and may underlie why CLD in HBV carriers waxes and wanes over many years. In addition, HBxAg-mediated changes in host gene expression that promote the cell cycle may also contribute to alterations in the state of hepatocellular differentiation, which also has an impact upon virus gene expression and replication during the course of chronic infection. For example, in HBV infection, there is an inverse correlation between the levels of viral replicative forms in the liver along with virus titers in serum and the progression of CLD. Most people in the early years of chronic infection have high levels of virus replication in both the liver and serum, while the great majority of people with cirrhosis or HCC have little or no evidence of virus replication (12) (Figure 1). There is, however, a direct correlation between the levels and distribution of intrahepatic HBxAg expression and the progression of CLD (35, 36, 41, 42). Interestingly, patients who are carriers for a few years tend to have a normal liver composed mostly of quiescent, differentiated hepatocytes. In contrast, carriers with cirrhosis or HCC tend to have livers with many cells undergoing regeneration, where cells are not quiescent, nor fully differentiated. This is especially true for HCC cells, which are often undifferentiated and generally do not support HBV replication *in vivo* or when isolated as corresponding dedifferentiated hepatoma cell lines *in vitro*. Studies with the related duck hepatitis B virus (DHBV) have also shown an inverse correlation between virus replication and the state of hepatocellular differentiation, with freshly plated duck hepatocytes fully capable of supporting DHBV replication, but that as the primary hepatocytes dedifferentiate in culture, they lose their ability to replicate DHBV (62). Interestingly, treatment of DHBV infected animals with the hepatotoxic antiviral agent, 2'-deoxycarbocyclic guanosine, resulted in more rapid clearance than treatment with a nontoxic nucleoside analog, suggesting that hepatocyte proliferation and turnover may contribute importantly to virus clearance (63). This also appears to occur in acutely infected woodchucks (64), although the mechanism whereby the entire liver resolves the virus infection without a massive inflammatory response, remains to be adequately understood.

#### 5.3. HBxAg promotes cell cycle progression by blocking negative growth regulatory pathways

HBxAg also impacts upon the cell cycle by promoting the phosphorylation (inactivation) of the retinoblastoma gene product (65, 66), perhaps through the

## HBxAg and cell cycle progression

activation of cdk2 and cdc2 (56), resulting in the release of E2F1, and stimulated entry into the cell cycle (67) (Figure 1). Interestingly, E2F1 binds to and inactivates the tumor suppressor, p53 (68) or may cooperate with p53 to trigger apoptosis (69). Normally, E2F1 activates the p53 promoter, thereby providing a negative regulatory feedback loop on cell cycle progression (70). In this regard, the fact that HBxAg also binds to and functionally inactivates p53 by cytoplasmic sequestration (71), and inhibits the p53 promoter (70), may shift the outcome of E2F1 activation in virus-infected cells. In addition, E2F1 cooperates with HBxAg in the stimulation of the Rb promoter (72), resulting in overproduction of Rb, which in turn suppresses apoptosis (73). Decreased Rb-E2F1 complexes in HBxAg positive hepatocytes may also relieve G<sub>1</sub> arrest triggered by transforming growth factor beta 1 (TGFbeta 1), contact inhibition, or and cdk inhibitor p16<sup>INK4a</sup> during chronic infection (74). The fact that HBxAg confers TGFbeta 1 resistance (75) and overcomes contact inhibition by promoting the growth of liver cells in soft agar (44-46), illustrates how alterations in the cell cycle alter hepatocellular growth and survival that are important to the pathogenesis of HCC

### 5.4. Beta-catenin activation in promoting cell cycle progression in tumor development

The recent finding that Wnt/beta-catenin signaling is stimulated by HBxAg (76), that activated beta-catenin stimulates expression of cyclin D1, and that cyclin D1 in complexes with cdk4 or cdk6 stimulate the E2F1 promoter (77), provides another pathway whereby HBxAg may stimulate the cell cycle (Figure 1). Independent observations that is constitutively activated in more than 20% of HCCs by mutation (78, 79), and that elevated cyclin D1 expression is associated with hepatocarcinogenesis in mouse and human HCC (80, 81), suggests that this mechanism may be operative *in vivo*. Interestingly, mutant normally induces the accumulation and activation of p53 as a homeostatic mechanism to prevent cancer (82), but in the presence of HBxAg, p53 is functionally inactivated by direct binding (83, 84) and transcriptional repression (85), so that the constitutive activation of beta-catenin target genes, such as *myc* and cyclin D1, contribute importantly to the development and progression of cancer. Importantly, the up-regulated expression of mutant or wild type in human HCC (86), combined with the activation of *myc* and cyclin D1 in tumor bearing HBV carriers (86, 87) and in transgenic mice developing HCC (88), further suggest constitutive Wnt signaling plays an important role in the pathogenesis of HCC. The fact that HBxAg activates ras signaling (56), and that activated cooperates with ras in transformation (82), provides a putative mechanism whereby the HBxAg mediated functional inactivation of p53 contributes to constitutive oncogene activation and tumor formation. The fact that both HBxAg and ras also stimulate the expression of the oncogene, *mdm-2* (89, 90), which targets p53 for proteasome mediated degradation, further emphasizes that inactivating p53 by these multiple pathways, is a central element as to how HBxAg contributes to cell cycle progression and the development of HCC on the molecular level (Figure 1). However, beta-catenin activation is not

always associated with HCC development, since *c-myc*/transforming growth factor alpha transgenic mice developed HCC characterized by extensive genomic instability, LOH among many chromosomes, and a low rate of beta-catenin activation. In contrast, *c-myc*/E2F-1 transgenic mice developed HCC characterized by a high frequency of beta-catenin activation in the absence of extensive genomic instability and LOH at other sites (91). The levels of intracellular HBxAg, state of cellular growth and differentiation, and the expression levels of HBxAg binding partners in the infected hepatocyte may influence which of these mechanisms predominate in tumor development.

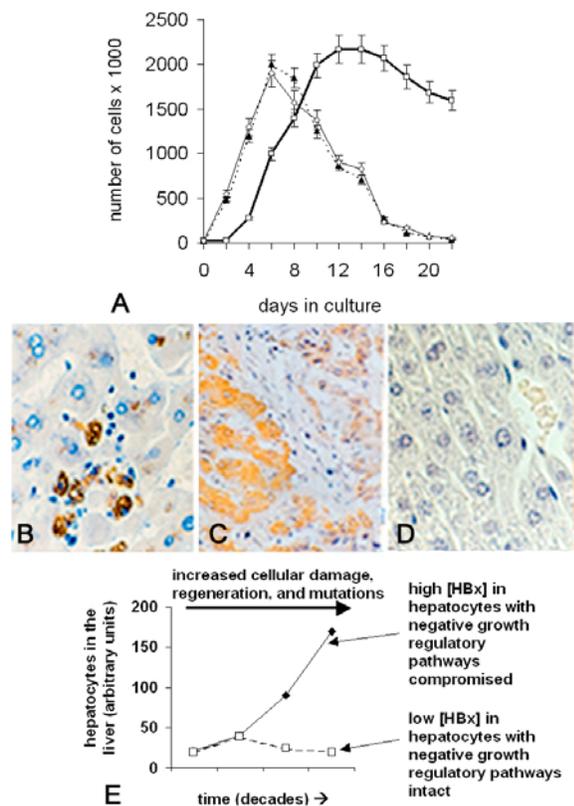
### 5.5. HBxAg promotes cell cycle progression by modulating apoptotic signaling

The fact that HBxAg promotes hepatocellular growth *in vitro* and tumorigenesis *in vivo*, suggests that it has a major impact upon cell cycle control in the pathogenesis of HCC. This may occur if HBxAg down-regulates the expression and/or blocks the function of molecules in apoptotic pathways. The finding that HBxAg binds to and functionally inactivates the tumor suppressor, p53, both *in vitro* and *in vivo* (83, 84), and that such complex formation is associated with the development of HCC in HBx transgenic mice (92), suggests that the inactivation of p53 plays an important role in releasing the brakes on cell cycle and growth control. The finding that HBxAg binds to and inactivates essentially all of the wild type p53 in HBx transgenic mice that develop HCC (92), and that HBxAg is also in complexes with wild type p53 in the majority of human HCCs (93), further suggest that the inactivation of wild type p53 is an important step in the deregulation of growth control in HCC.

In the context of CLD, where oxidative radicals in the inflammatory process result in DNA damage, as well as in liver cell destruction and regeneration, p53 expression is often triggered to shut down hepatocellular growth. However, when this happens in an HBV infected liver, the p53 response may be neutralized by HBxAg. Given that HBxAg also stimulates a number of signaling pathways that promote hepatocellular growth and survival (94, 95), it is no surprise that HBxAg stimulates cell cycle progression, in part, by binding to and functionally inactivating wild type p53. Interestingly, the tumor suppressor PTEN, which is transcriptionally activated by p53 (96), is also depressed in HCC by HBxAg (97). The loss of PTEN, which is a phosphatase that normally inhibits PI-3K activity, results in constitutive PI-3K activity, which promotes hepatocellular proliferation (98). In addition, HBxAg inhibits expression of the p53 effector, p21<sup>WAF1/CIP1/SDI1</sup> at the transcriptional level at p53 dependent and independent sites within the *p21* promoter (99, 100).

The above evidence suggests the centrality of lost p53 function to tumor development. However, the induction of HBxAg expression in several different liver cell lines resulted in apoptosis, not proliferation (101, 102). Independent evidence has shown that HBxAg sensitizes mouse fibroblasts to p53-mediated apoptosis after exposure to DNA damaging agents (103). HBxAg triggered

## HBxAg and cell cycle progression



**Figure 2.** Growth curves for WB-F344 control and WB-F344X cells. **A.** Control WB-F344 cells (○), WB-F344X clones 3.8 (Δ) and 4.13 (▲). The expression plasmid, pCMV-HBx or pCMV vector were used to stably transfect WBF344 cells, and individual colonies were selected in G418. For each colony,  $2 \times 10^4$  cells/well were plated in 30-mm wells. Media was changed daily, and viable cells were determined by trypan blue exclusion and independently, by MTS assay, in triplicate wells for two control and two HBxAg expressing clones at the time points indicated. **B-D.** Immunohistochemical staining for HBxAg in the liver of a patient with **(B)** chronic hepatitis (x400), **(C)** with cirrhosis (dark staining on the left half of the panel) and HCC (lighter staining on right half of panel) (x200), or **(D)** in uninfected liver (x400). Note in panel **B** that degenerating hepatocytes are strongly HBxAg positive, and yet in panel **C**, there is no evidence of degeneration in peritumor hepatocytes, despite the fact that they are HBxAg positive. **E.** Diagram of model outlined in the text. When HBxAg is expressed in hepatocytes with intact negative growth regulatory pathways, the cellular response to HBxAg may be growth arrest or apoptosis. As these negative growth regulatory pathways are inactivated by mutations, methylation, or other mechanisms, HBxAg promotes increased cell proliferation, which contributes importantly to the development of HCC. See the text for additional details.

apoptosis in NIH3T3 cells suppressed growth in soft agar, while HBxAg triggered apoptosis in Chang liver cells inhibited growth in 1% serum (104). In addition, in some strains of HBx transgenic mice, expression of HBxAg is associated with spontaneous apoptosis, which is p53

independent, suggests that HBxAg could trigger apoptosis *in vivo* (102, 105). Although this suggests that other HBxAg triggered pathways may be more important in triggering cell cycle progression, the accelerated growth following the onset of HBxAg expression is known to trigger a corresponding cellular response, part of which includes the induction and accumulation of p53. Under these circumstances, if p53 induction is not completely inactivated by HBxAg (i.e., if intracellular concentrations of HBxAg are not high enough), then p53 may trigger cell cycle arrest and/or apoptosis. This has been observed in the immortalized but nontumorigenic rat liver epithelial cell line, WBF344, where the introduction of HBxAg results in an initial period of very rapid cellular growth, followed by induction of p53 and the cyclin dependent kinase inhibitor, p21<sup>WAF1/CIP1/SDI1</sup> (i.e., the cellular response), and finally by massive apoptosis compared to HBxAg negative cells growth in parallel (Figure 2A). As outlined below, cell fate may be dependent upon the intracellular concentration of HBxAg. If not all of the induced negative growth regulatory proteins are neutralized by HBxAg, then HBxAg positive cells would correlate with cells undergoing degeneration (Figure 2B). If enough intracellular HBxAg is present, and/or the cellular response to HBxAg is not as vigorous due to mutation or inactivation of these negative growth regulatory pathways, then high concentrations of intracellular HBxAg could result in tumor formation (Figure 2C). This is consistent with observations that HBxAg expression is highest and most widespread in cirrhotic and dysplastic nodules from which HCC develops (35), and in peritumor tissue (44-46) (Figure 2C).

To expand upon this model, the functions of HBxAg in the stimulation of the cell cycle and cell growth versus cell cycle arrest and apoptosis, seem to depend upon the cell type that HBxAg is being expressed, the state of cellular differentiation, and the intracellular concentration of HBxAg. For example, NIH3T3 cells co-transfected with HBxAg and one of several oncogenes, resulted in apoptosis (106). The introduction of HBxAg also sensitized Chang liver cells to apoptosis when cells were cultured in 1% calf serum (106) or treated with tumor necrosis factor alpha (TNF alpha) (107). Increased sensitivity to TNF alpha killing was associated with a prolonged stimulation of N-myc and the stress-mediated mitogen activated-protein kinase 1 (MEKK1) pathway. Others have also shown that HBxAg causes cell cycle arrest at the G1/S transition, suppresses colony formation in culture, and induces apoptosis in a variety of cell lines (66). Using a series of HBxAg mutants, further work has shown that the ability of HBxAg to trigger apoptosis correlates with the ability of HBxAg to mediate *trans*-activation (108, 109). On the other hand, the findings that HBxAg blocks p53 (71, 110), Fas (111), TNF alpha (112), and TGFbeta 1 (75) apoptosis, as well as caspase 3 activity (113), demonstrates that HBxAg may promote cell cycle progression, in part, by inhibiting apoptotic responses. Given that the *trans*-activation function of HBxAg also correlates with its ability to mediate transformation (44-46), the cellular response to HBxAg mediated *trans*-activation is likely to be central to the regulation of apoptosis/growth and to cell fate during chronic infection. Hence, HBxAg strongly stimulates the

## HBxAg and cell cycle progression

cell cycle and cell growth. In normal cells, with intact negative growth regulatory pathways, these pathways are triggered in response to HBxAg, resulting in either growth arrest and/or apoptosis (Figure 2E). In cells where one or more negative growth regulatory pathways are compromised by point mutation, LOH, methylation or by other mechanisms, HBxAg continues to promote growth and tumorigenesis instead of growth arrest and apoptosis (Figure 2E). This model is supported by observations in HBx/*c-myc* transgenic mice, where hepatocytes were mitotically active and proliferated soon after birth, but this high mitotic index was accompanied by a high rate of apoptosis, which later declined as HCC developed (114). Interestingly, when HBx transgenic mice were subjected to partial hepatectomy, or when hepatocytes from HBx transgenic mice were transplanted into nontransgenic littermates, liver regeneration was inhibited (115). Further work showed that the inhibitory activity consisted of one or more factors in the serum of HBx transgenic mice. A major candidate, TGFbeta 1, which is known to inhibit liver regeneration following partial hepatectomy, was not elevated in serum of HBx transgenic mice. However, the recent finding that HBxAg potentiates TGFbeta 1 activity by suppressing expression of the TGFbeta 1 inhibitory binding protein, alpha 2-macroglobulin (116), suggests that altered TGFbeta 1 activity (but not total levels of TGFbeta 1) may indeed be a paracrine-based mechanism whereby HBxAg inhibits liver regeneration (Figure 1). If high concentrations of HBxAg in dysplastic hepatocytes or early HCC inhibit hepatocellular regeneration in the context of CLD, it would favor the survival and outgrowth of tumor cells.

### 5.6. HBxAg binding partners and cell cycle progression

In addition to a cellular response to HBxAg, the ability of HBxAg to trigger growth or apoptosis may depend upon the state of HBxAg polypeptide aggregation (117) and/or its' binding to other cellular proteins. Since the primary sequence of HBxAg contains more than 60% hydrophobic residues, aggregation and corresponding subcellular localization of HBxAg may alter its functional properties. For example, cytoplasmic HBxAg may alter gene expression by promoting the constitutive activation of NF kappaB, MAPK, src kinases, PI3K and other signaling pathways, while nuclear HBxAg may alter gene expression by binding to a number of transcription factors (118). Interestingly, cytoplasmic HBxAg appears to stimulate hepatocellular growth and survival, while nuclear HBxAg appears to support HBV gene expression and replication at the level of the virus minichromosome (119, 120), which is the template for all of the virus mRNAs, including the pregenomic RNA which is central for hepadnavirus replication (121). The ability of HBxAg to stimulate the cell cycle may also depend upon its binding partners in the cell. For example, HBxAg binds to and sequesters p53 (92) and the senescence factor, p55<sup>sen</sup> (122) in the cytoplasm, while the binding of HBxAg to transcription factors recruits HBxAg to the nucleus. These observations underscore the potential importance of HBxAg, as a protein that acts by binding to other proteins (and itself), upon the ability of HBxAg to regulate cell cycle progression.

The ability of HBxAg to stimulate the cell cycle

is also likely to be dependent upon whether it triggers hepatoprotective signaling pathways during chronic infection. For example, HBxAg is known to stimulate NF kappaB (123, 124), which is hepatoprotective in the face of the oxidative damage that often accompanies CLD. The extent of HBxAg mediated NF kappaB activation is likely to be influenced by the intracellular concentration of HBxAg, how much of it binds to I kappaB (125), and the extent of ROI in the liver, among other factors. Strong NF kappaB activation will contribute to blocking the proapoptotic cellular response to the presence of HBxAg (126) and in promoting cell growth and survival (127). One way that HBxAg may trigger a hepatoprotective response is by binding to an outer mitochondrial voltage-dependent anion channel, HVDAC3, which results in oxidative stress and altered mitochondrial membrane potential (128), the release of ROI from mitochondria, and the subsequent activation of NF kappaB and STAT-3 (129). Since NF kappaB and STAT-3 motifs are found in a wide variety of cellular genes that promote cell growth and proliferation, and are important for DNA replication and repair, these observations provide a link between HBxAg, ROI and carcinogenesis. In another example, HBxAg stimulates the levels and activity of TGFbeta 1 (116, 130), which is usually proapoptotic, but the HBxAg activation of multiple hepatoprotective pathways (e.g., containing NF kappaB, MAPK, and PI3K) override the negative growth regulatory signals from TGFbeta 1. The consequence of these events is to favor the growth and survival of HBxAg positive hepatocytes at the expense of uninfected hepatocytes during chronic infection (Figure 1). Under such circumstances, the proportion of HBxAg positive hepatocytes in the liver would increase with each bout of CLD and subsequent regeneration. Given that HBV DNA integration occurs most readily during regeneration, resulting in increased accumulation of intracellular HBxAg over time, it is not surprising that HBxAg is such a prevalent marker in the livers of HBV carriers with cirrhosis (35) (Figure 1), and that sustained high levels of intracellular HBxAg is an important element in X transgenic mice that develop HCC (50, 92).

### 5.7. HBxAg activation of signaling pathways involved in cell cycle progression may be related to state of cellular differentiation

The ability of HBxAg to modulate the cell cycle also appears to be sensitive to the state of hepatocellular differentiation. Introduction of HBxAg into immortalized but untransformed AML-12 cells (originally isolated from TGF alpha transgenic mice), in which HBxAg expression was under control of the inducible tetracycline promoter, resulted in a number of clones with distinct characteristics upon induction of HBxAg. Only clones that displayed hepatocyte specific markers (i.e., markers of hepatocellular differentiation) were transformed upon induction of HBxAg expression characterized on the molecular level by prolonged induction of ATF3, c-fos (131), ras-raf-MAPK (132, 133), cyclins D1, A and B1, as well as cdk2 kinase (134). Independent observations have shown that the HBxAg induction of ras signaling, and parallel activation of cyclins A and B, deregulate cell cycle checkpoints and promote growth in serum free medium (56). The fact that HBxAg stimulates ras by functioning as an intracellular

## HBxAg and cell cycle progression

cytoplasmic activator of the src family of tyrosine kinases (135) suggests that HBxAg may promote growth and survival in serum free medium by constitutively activating src in the absence of growth factor bound activation of receptor tyrosine kinases, which transiently stimulate src signaling in uninfected cells. In contrast, clones untransformed by HBxAg appeared to be less differentiated, only had transient up-regulated c-fos expression, and had constitutive activation of the JNK pathway (132). Untransformed AML12 cells also had elevated expression of p21<sup>WAF1/CIP1/SDI1</sup>, the tumor suppressor, p19<sup>ARF</sup>, and the proapoptotic genes bax and insulin-like growth factor binding protein-3 (IGFBP-3), although no G<sub>2</sub>/M dependent apoptosis was observed in HBxAg expressing compared to negative untransformed cultures. These results confirm that HBxAg deregulates the G<sub>2</sub>/M checkpoint in differentiated compared to undifferentiated immortalized hepatocytes, which in some respects mimics the events in HBxAg cirrhotic and dysplastic nodules. Further, the independent finding that HBxAg expression in primary mouse hepatocytes results in increased p21<sup>WAF1/SDI1/CIP1</sup> and p27<sup>Kip-1</sup> expression and growth arrest, while the introduction of HBxAg into p21<sup>-/-</sup> hepatocytes results in a weak stimulation of DNA synthesis (136), is also consistent with the idea that the cellular response to HBxAg is an important determinant of cell fate (Figure 2E).

### 5.8. HBxAg triggered genetic instability may promote cell cycle progression

HBxAg also appears to regulate cell growth and apoptosis by altering genetic stability. This appears likely, considering that HBxAg binds to and functionally inactivates p53 (83, 84), and that it suppresses the promoters of *p53*, *Rb* and p21<sup>WAF1/CIP1/SDI1</sup> (70, 72, 85, 100, 137), which compromises genome integrity while promoting cell cycle progression. This is highlighted by the finding that HBxAg promotes chromosomal rearrangements and micronuclei formation in HepG2 cells (138). Interestingly, HBxAg has a leucine-rich nuclear export signal (NES) which binds to the nuclear export receptor, Crm1, which is responsible for the cytoplasmic localization of HBxAg (139). HBxAg sequesters Crm1 in the cytoplasm of tissue culture cells and in HCC tissue (139). This is associated with the nuclear translocation and activation of NF kappaB (139), MAPK and mdm-2, suggesting that HBxAg may stimulate cell cycle progression and oncogenesis by blocking the nuclear export (i.e., blocking sequestration) of molecules that promote tumorigenesis. Further work has revealed that the cytoplasmic sequestration of Crm1 by HBxAg is associated with the development of multi-polar spindles and aneuploidy (140), resulting in an increased frequency of defective mitoses and chromosomal transmission errors, which contribute importantly to genome instability in the early steps of carcinogenesis.

Another way that HBxAg may compromise genome integrity is by binding to a UV-damaged DNA binding protein (UV-DDB), which may compromise DNA repair (141) (Figure 1). Mutational analysis of HBxAg has shown a partial correlation between the reduction of repair

activity in HBxAg positive cells with the ability of HBxAg to bind UV-DDB (142). Several studies have also shown that HBxAg-DDB complex formation results in the accumulation of HBxAg in the nuclei of infected cells, which correlated with increased HBxAg *trans*-activation function, with apoptosis (143), and with loss of cell viability (144) (the latter may be a cellular response to increased HBxAg *trans*-activation function). In the context of liver regeneration, DDB becomes localized to the nuclei of hepatocytes during the late G<sub>1</sub> phase of the cell cycle, followed by a sharp decrease in progression through the G<sub>1</sub>/S checkpoint (145). The finding that HBxAg mutants that do not bind DDB do not trigger apoptosis (143), suggests that cell fate may also depend upon the nature of the X protein that is made from integrated templates. It turns out that when the X region of HBV integrates into host DNA during CLD, the HBxAg polypeptides made are often C-terminally truncated, accumulate point mutations at other sites, and lack anti-proliferative (or proapoptotic) activities in tissue culture cells (66, 146). Importantly, the extent of mutations in the X region was far greater in tumor compared to nontumor liver cells from the same patients (146, 147), suggesting a link between compromised DNA repair, failure to trigger apoptotic responses, and uncontrolled cell cycle progression. Many of these mutants retained their ability to bind and functionally inactivate p53 (148), again suggesting that the escape from apoptosis is an important feature in the pathogenesis of HCC.

### 5.9. HBxAg and oncogene activation in cell cycle progression

The ability of HBxAg to stimulate cell cycle also appears to correlate with its ability to constitutively activate the expression of several oncogenes. Although early studies did not show elevated oncogene expression in HCC (149-151), later work showed that the cellular genes targeted by HBV in HCC are key regulators of cell viability and proliferation (152). For example, the fact that HBxAg is strongly expressed in peritumor tissue compared to HCC cells (36), that HBxAg constitutively activates the ras-raf-MAPK pathway (133), which in turn, transcriptionally up-regulates the expression of *c-myc* (132, 133), suggests that *c-myc* over-expression is an early event in hepatocarcinogenesis. This is further supported by the finding that *c-myc* is a transcriptional target of HBxAg (153), and that Wnt (beta-catenin) signaling is activated in HBxAg expressing cells (76). In addition, the oncogene, mdm-2, appears to be over-expressed in tumor (154, 155), and is related to metastasis (156, 157). The finding that HBxAg *trans*-activates mdm-2 (90) provides further evidence that HBxAg promotes constitutive oncogene activation, which in this case, occurs in the context of tumor progression and invasiveness. Finally, recent work has shown that HBxAg up-regulates the expression of a novel gene, URG4, which promotes the growth of HepG2 cells in culture and in soft agar, and accelerates tumor formation of HepG2 cells in nude mice (44), although the mechanism(s) whereby this occurs remain to be investigated. However, it is clear that part of the way HBxAg stimulates cell cycle progression is by its ability to up-regulate the expression of multiple oncogenes.

**6. SUMMARY AND FUTURE PROSPECTS**

The above discussion helps to explain how HBxAg contributes to the development of the liver cancer. It underscores that the apparently proapoptotic and anti-apoptotic properties of HBxAg may have to do with the nature of the HBxAg polypeptides made during chronic infection (full-length or truncated), the partners that HBxAg binds to in the cell, the intracellular concentration of HBxAg, and the nature of the cellular response to HBxAg function. Given that HBxAg stimulates the cell cycle by a variety of different mechanisms (Figure 1), the cellular response to HBxAg likely contributes as to whether hepatocytes undergo growth arrest, apoptosis or enhanced survival and neoplastic growth. Given these observations, HBxAg contributes importantly to multi-step carcinogenesis, with cellular alterations being necessary but not sufficient for tumor development. These observations also reveal how the pleomorphic properties of HBxAg could result in different cell fates, depending upon the cellular environment in which HBxAg is expressed. Most importantly, the data in this review suggests that HBxAg is an important target for antiviral drug discovery and for the development of novel approaches against HCC. Finally, given that HBxAg brings about these changes in the liver years before the appearance of HCC, provides multiple opportunities to develop chemopreventative approaches that may alter the course of chronic infection and prevent the development of one of the most frequent and deadly cancers in the world.

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**Send correspondence to:** Dr Mark A Feitelson, PhD, Room 222 Alumni Hall, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107 USA, Tel: 215-503-1036, Fax: 215-503-9982, E-mail: Mark.Feitelson@jefferson.edu

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