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Review

Hepatitis B Virus x Antigen in the Pathogenesis of Chronic Infections and the Development of Hepatocellular Carcinoma

Mark A. Feitelson* and Ling-Xun Duan†

*From the *Department of Pathology, Anatomy, and Cell Biology and †Dorrance H. Hamilton Laboratories, Center for Human Virology, Division of Infectious Diseases, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania*

Chronic infection with hepatitis B virus is associated with a high incidence of liver diseases, including hepatocellular carcinoma. Hepatitis-B-virus-encoded X antigen (HBxAg) stimulates virus gene expression and replication, which may be important for the establishment and maintenance of the chronic carrier state. Integration of viral DNA encoding HBxAg during chronic infection results in increased X antigen expression. HBxAg overexpression may alter signal transduction pathways important for the regulation of cell growth during hepatocellular regeneration. The finding that HBxAg binds to and inactivates negative growth-regulatory molecules, such as the tumor suppressor p53, suggests additional ways that HBxAg may act in hepatocarcinogenesis. HBxAg may also stimulate the expression of positive growth regulators, such as insulin-like growth factor II and the insulin-like growth factor I receptor. The finding that HBxAg may compromise DNA repair and that it may effect the normal turnover of growth-regulatory molecules in the proteasome may also contribute to its carcinogenic properties. Hence, HBxAg may contribute to the pathogenesis of chronic infection and development of hepatocellular carcinoma in a variety of ways. (Am J Pathol 1997, 150:1141–1157)

An estimated 300 million hepatitis B virus (HBV) carriers worldwide[1] are at increased risk for the development of chronic active hepatitis (CAH), cirrhosis, and hepatocellular carcinoma.[2,3] Although a highly efficacious vaccine for HBV is now available,[4] recurring bouts of CAH and the development of cirrhosis among chronic carriers, combined with the relatively few treatment options available,[5] continue to present formidable threats to public health. HBV-associated HCC is among the 10 most frequent cancers worldwide. At least 250,000 cases of HCC are diagnosed annually[6]; less than 3% of these patients survive 5 years. The relative risk of HBV carriers developing HCC approaches 200:1, which is one of the highest relative risks known for a human cancer.[2,7,8] Given the high frequency and mortality of HCC worldwide, elucidation of the mechanism(s) whereby HBV brings about chronic liver diseases, including HCC, will have a profound impact on the prevention and treatment of chronic HBV infections.

Association of HBV with Chronic Liver Diseases, Including HCC

There are multiple lines of evidence that HBV and related hepadnaviruses[9] in nature are major etiological agents of chronic liver diseases, including HCC. 1) For example, chronic liver diseases and HCC appear only after the appearance of HBV markers in blood,[2,10] suggesting cause and effect. 2) Among

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Address reprint requests to Dr. Mark A. Feitelson, Room 222, Alumni Hall, Department of Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107-6799.

human carriers, HCC often appears after episodes of CAH and the development of cirrhosis.[11] The identification of the carrier state and CAH as major risk factors for the development of HCC has been shown to be statistically significant in independent studies,[3,7] suggesting that HBV replication, gene expression, and hepatocellular turnover are critical factors in the pathogenesis of HCC. 3) Chronic infection of woodchucks with the related woodchuck hepatitis virus (WHV) results in CAH and HCC in up to 100% of carrier animals 2 to 3 years after infection.[12,13] HCC has also been observed in up to 30% of ground squirrels chronically infected (for 4 to 5 years) with the related ground squirrel hepatitis virus.[14] Infection of woodchucks with GSHV, however, resulted in only about 40% of the animals developing HCC at 4 to 5 years of age,[15] suggesting that the differences in tumorigenicity are in the viruses themselves, and that these viruses make a genetic contribution to HCC. The finding that WHV is a complete carcinogen in experimentally infected animals[13] further underscores the etiological relationship between chronic infections and the development of HCC. 4) Most HCC tissues and derived cell lines from chronically infected patients and woodchucks contain detectable virus antigens,[16–18] the expression of which may contribute to the development of HCC. Accumulating evidence suggests that expression of the X antigen is particularly relevant (see below), although the mechanism(s) by which HBxAg mediates transformation are still not well understood. 5) HBV and WHV DNA have been found integrated, often highly rearranged, at one or more sites within host DNA in tumors and tumor-derived cell lines.[6,19–22] These observations suggest that chronic hepadnavirus infections are associated with genetic instability. Overall, then, the balance of evidence strongly supports a close association between chronic hepadnavirus infection and the development of HCC.

Putative Roles for X Antigen in the Development of Chronic Infections and Chronic Liver Diseases

It is generally thought that the pathogenesis of CAH is mediated by immune responses against virus-infected cells.[23] Accordingly, the importance of different virus antigens as immunological targets may vary during the course of chronic infection. For example, during the first few years, chronic infection is often characterized by high levels of virus replication, suggesting that immune responses would be directed against the corresponding virus gene products in cells that support virus replication (Figure 1).[17,18,24] These potential targets include epitopes from all of the gene products of HBV, since products from each of the four virus genes are expressed during the period of virus replication. After virus is cleared from serum and liver, the persistence of CAH, as well as its progression to cirrhosis and HCC, would be expected to be associated with immune responses directed against virus gene products expressed from fragments of virus DNA that have become integrated into host chromosomal DNA. Integration is thought to occur most readily during hepatocellular regeneration, which follows each bout of hepatitis (Figure 1).[25,26] The role of hepatocellular destruction and regeneration in the development of HCC is strongly supported by epidemiological studies that have identified the most important risk factors for HCC as the chronic carrier state and the presence of chronic liver disease[2,7] and by documentation from several transgenic mouse systems in which HCC develops in association with prolonged periods of hepatocellular regeneration.[27,28] Hepatocellular destruction and regeneration may also provide the basis for the accumulation of genetic mutations, which contribute to multistep hepatocarcinogenesis.[6,29] Hence, the persistence of virus antigens in the liver and their immunological recognition make up important elements that contribute to the pathogenesis of HCC.

The finding that HBV (and WHV) DNA fragments are integrated into the cellular DNA of carriers[6,19–21,30–32] suggested that viral integration at one or a few sites in host DNA may result in the deregulated expression of oncogenes and/or tumor suppressor genes. This model seems to hold true for WHV-mediated carcinogenesis, in which *cis*-activation of N-*myc* and c-*myc* oncogenes by promoter insertion of viral DNA in or around these genes seems to be a common feature.[33–35] In HCCs associated with ground squirrel hepatitis virus infections, overexpression of the c-*myc* gene was caused by gene amplification and not promoter insertion.[36] However, the apparently random patterns of HBV integration into host DNA makes it unlikely that *cis*-acting mechanisms are common features of HBV-associated hepatocarcinogenesis, although integration near V-*erb*-A, cyclin A, or the retinoic acid receptor genes, for example, has been reported.[37–39] In addition, HBV integration has been associated with the loss of heterozygosity in many chromosomes,[30–32] suggesting that integration promotes genomic instability,[40] the latter of which is a common feature of carcinogenesis. Hence, although these results support the idea of multiple mechanisms whereby hepadnaviruses bring about hepatocellular transfor-

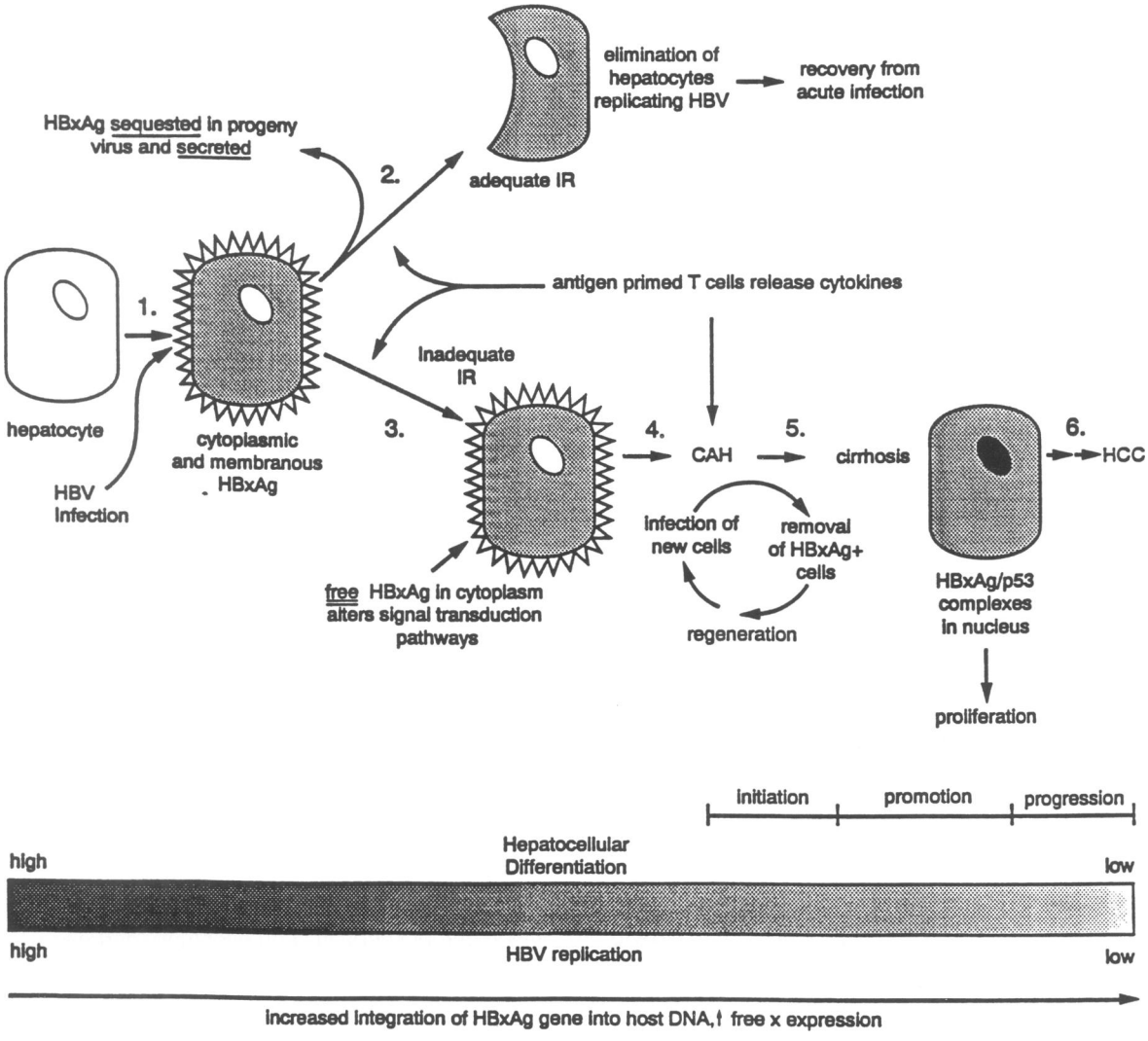

Figure 1. *Pathogenesis of chronic HBV infection. Susceptible hepatocytes are infected with HBV* (1) *and express HBxAg mostly in the cytoplasm* (gray) *and membranes* (spikes). *Most HBxAg is associated with virus replication complexes in the cytoplasm of infected cells.* (2) *In the presence of appropriate immune responses (IRs), the cells supporting virus replication are eliminated, resulting in the recovery from acute infection. In the event that immune responses are inadequate or inappropriate or do not appear soon enough* (3), *the virus spreads throughout the liver, and the chronic carrier state is established* (4). *Cytotoxic cytokines target and remove some of the virus-infected cells. This process is then followed by regeneration and infection of new cells, and the cycle is repeated many times during the course of chronic liver disease* (5). *The alteration of signal transduction pathways by HBxAg and the corresponding changes in host gene expression patterns may yield an increasing number of HBxAg-positive hepatocytes with phenotypes that are resistant to cytokine-triggered apoptosis and less able to support virus replication. The continued accumulation of intracellular HBxAg eventually inactivates negative growth-regulatory pathways, as examplified by its binding to p53, resulting in proliferation and accumulation of mutations in the place of controlled regeneration and appropriate DNA repair. It is proposed that such cells are most prone to develop into HCC as additional hits accumulate* (6).

mation, further research is beginning to reveal common denominators in these different systems.

Although the sites of viral integration with regard to the host genome seem to be random, there are preferred sites within the viral genome. During virus replication, the origin of replication for each viral DNA strand consists of an 11-bp direct repeat sequence.[41] Since the direct repeat sequences are at the end of the growing linear viral DNA strands and overlap the X gene of the virus, the X region becomes the most frequently integrated sequence (Figure 2). Most primary tumors and tumor-derived cell lines have some or all of the X region and upstream pre-S/S sequences integrated.[32] In addition, most primary tumors made X region transcripts, whereas relatively few made transcripts from other regions of the genome.[42,43] Many of these integrated fragments make hepatitis-B-virus-encoded X antigen (HBxAg) capable of *trans*-activation both *in vitro*[44,45] and *in vivo*[46] although the natural targets of HBxAg *trans*-activation in liver diseases, including HCC, remain to be clearly identified. Furthermore, immuno-

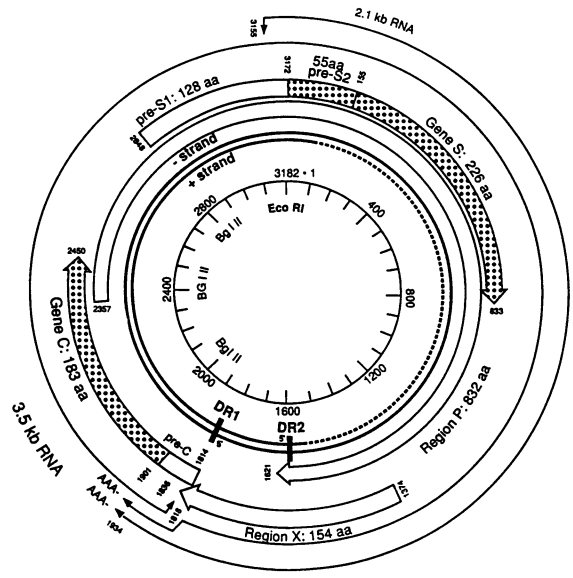

Figure 2. *Diagram of the HBV genome showing the direct repeat (DR) sequences at the end of each viral DNA strand. Most integration occurs at DR1, with the integrated template containing most or all of the X region sequences and, in many cases, the upstream S and pre-S/S gene sequences as well. The proximity of the X region to the end of the viral genome that integrates into host DNA suggests that this region will integrate most often, the upstream pre-S/S region less often, and the core region only rarely. These observations may provide the rationale for the patterns of virus gene expression in chronically infected livers most often seen among patients with chronic liver disease who are no longer replicating HBV.*

histochemical staining of livers from more than 200 carriers with CAH or cirrhosis and from more than 100 carriers with HCC demonstrated that HBxAg was more prevalent than the other gene products of HBV.[25,47] In many cases, it was the only HBV antigen detectable by staining. The close correlation between X antigen staining and hepatitis in chronically infected woodchucks,[26] and its persistence at high levels in the livers of X transgenic mice that go on to develop liver tumors,[48–50] also suggest that the persistent expression of HBxAg is consistent with the development of HCC. Hence, a common feature of pathogenesis in chronic HBV infection is not where the viral DNA integrates, but that in most cases the integrated sequences make HBxAg. Recent observations have also detected X antigen in chronically infected livers of woodchuck carriers that develop HCC,[51] suggesting that here, too, the persistent expression of X antigen is associated with the carrier state and the development of associated chronic liver diseases.

HBxAg has been widely studied as a *trans*-activating protein that seems to act promiscuously in the stimulation of a variety of virus and host gene promoters.[52] It has been suggested that the pleiotrophic effects of HBxAg on transcription may reflect its binding to a variety of other transcription factors, thereby acting as a coactivator in infected cells.[53,54] The consequence of HBxAg transcriptional coactivation during infection may be to stimulate HBV gene expression and replication,[55,56] which would be important for the establishment and persistence of virus replication during chronic infection. The finding that X-negative WHV DNA is not capable of establishing the chronic carrier state in experimentally infected animals,[57,58] combined with the finding that HBV with naturally occurring deletions within the X gene is the predominant viral genotype in patients with HBV-seronegative non-A, non-B hepatitis,[59] also suggests that the persistent expression of X antigen may be required for the establishment and maintenance of the chronic carrier state.[60] During the period of virus replication, however, HBxAg is either sequestered in the replication complex of the virus[61,62] or secreted into serum as a soluble polypeptide, in which it often appears with HBeAg and virus particles (Figure 1).[63,64] In hepatocytes supporting virus replication, then, the amount of HBxAg accumulating intracellularly is relatively low (Figure 1).[47] Under these circumstances, the major roles of HBxAg seem to be in the support and maintenance of virus replication. Hence, one way HBxAg may contribute to the development of HCC is by helping to maintain the chronic carrier state and, in doing so, may provide the environment (ie, cells replicating virus and expressing virus antigens) in which chronic hepatitis and eventually cirrhosis may develop.

The inverse correlation between HBxAg staining in the liver and markers of virus replication in the serum suggests that HBxAg could be made independently from virus replication, probably from integrated templates of viral DNA (Figure 1).[65] The immunological recognition and removal of cells replicating virus, combined with the regeneration of cells harboring mostly or entirely integrated HBV DNA, results in the clonal expansion of hepatocytes.[19] The expansion of HBxAg-positive cells in the liver by continued viral DNA integration and clonal expansion and the increased expression levels of HBxAg, which are likely to accompany such expansion,[25,47] result in a liver increasingly dominated by HBxAg-positive cells, which are then ripe for additional "hits" that contribute to the process of multi-step carcinogenesis. Proposed characteristics that allow for the outgrowth of HBxAg-positive cells include: 1) the direct stimulation of cell growth by HBxAg,[66–70] 2) the inactivation of negative growth regulators, such as tumor suppressor p53 by HBxAg,[71–73] 3) the increased resistance of HBxAg-positive cells to apoptosis mediated

by cytotoxic cytokines released during CAH, and 4) the apparent inactivation of one or more DNA repair pathways by HBxAg, which permits the accumulation of additional mutations in cellular DNA during regeneration.[72] The presence of HBxAg on or near the plasma membrane of hepatocytes from patients with chronic hepatitis and cirrhosis[47] is also consistent with it being a classical human-leukocyte-antigen-associated immune target, but there are no firm data to support this. In contrast, the lack of membranous staining and large increase in the nuclear staining of HBxAg among patients with cirrhosis and dysplasia[25,47] suggest that HBxAg may act as a promiscuous coactivator within liver lesions that are present just prior to the appearance of HCC. The fact that more than 95% of the patients with cirrhosis and dysplasia were HBxAg positive in the liver, compared with less than 70% of patients with HCC,[25] suggests that the action of HBxAg is important at the time that tumors appear, but that once tumors form, the actions of HBxAg are no longer rate limiting, and the antigen is no longer selected for among infected cells. Hence, an understanding of the role of HBxAg in the infected hepatocyte just prior to the appearance of a tumor should take into consideration the possibility that the effectors of HBxAg co-*trans*-activation in the nuclei of hepatocytes need to be identified.

It is clear that HBxAg is not an acutely transforming oncogene product, since HCC appears some 30 to 50 years after infection. Part of the reason for this may be that HBxAg is secreted into blood or sequestered in viral replication complexes, leaving little free HBxAg in the cell during the early phases of chronic infection (Figure 1). Low levels of free HBxAg in the cell also seem to promote the arrest of hepatocytes in the G_1 stage of the cell cycle (H. Reis, J. Pan, X. L. Duan, and M. A. Feitelson, unpublished results), which would favor virus replication even during a bout of CAH, in which virus-infected cells are being removed and the regenerating cells do not support virus replication.[74,75] Furthermore, the predominant cytoplasmic localization of HBxAg in hepatocytes during periods of infection characterized by CAH and cirrhosis may alter signal transduction pathways, which results in a correspondingly altered sensitivity of hepatocytes to the action of cytotoxic cytokines, such as increasing the resistance of infected hepatocytes to apoptosis[76] and/or temporaily downregulating virus gene expression in the presence of cytokines so as to allow infected cells to "escape" otherwise eliminating immune responses.[77,78] In contrast, the predominantly nuclear localization of HBxAg in the livers of patients with cirrhosis and dysplasia may alter patterns of host gene expression relevant to carcinogenesis. It is postulated that the uncoupling of HBxAg with virus replication during chronic infection results in increased levels of HBxAg only in cells in which some negative growth-regulatory circuits have already been inactivated, either through mutation or by the fact that regenerating hepatocytes in an increasingly abnormal liver architecture are no longer fully differentiated, as are resting hepatocytes in a normal liver. The evidence that is consistent with this idea is that high levels of HBxAg in normal hepatocytes inhibits cell growth (X. L. Duan and M. A. Feitelson, unpublished data), and that HBxAg is much more likely to stimulate DNA synthesis and unregulated growth only in cells that are already close to transformation. The latter point is exemplified by documentation that HBV DNA in general, and the X product in particular, can transform NIH 3T3 cells[66] and a mouse hepatocyte cell line, FMH202,[67,79] into lines that acquire the ability to grow as tumors in nude mice. The FMH202 line was also transformed following transfection with DNA from a human HCC nodule that contained integrated viral sequences.[80] Again, transformation was dependent on the presence of X gene sequences.[80] Subcellular fractionation of transformed FMH202 cells showed that HBxAg was detected exclusively in the cell nuclei,[67] which is consistent with its role as a transcriptional coactivator.[53,54] One possibility for the shifting patterns and properties of HBxAg may lie in the discovery and characterization of different X-binding proteins, which may alter both the subcellular localization and function of HBxAg at different points during infection.

HBxAg does not bind nucleic acids directly but instead seems to mediate its effects on cell behavior by protein-protein interactions.[81] The observation that HBxAg can bind to cAMP response element-binding protein, activating transcription factor-2,[81] Oct-1,[82] TATA-binding protein,[83] a subunit common to RNA polymerases,[84] and other elements of the transcriptional machinery,[53,54] combined with observations that such binding often results in altered DNA binding and activity of these components, provides a common denominator from which some of the promiscuous *trans*-activation properties of HBxAg could be explained. In the context of chronic infection, these interactions probably become important with the increasing intracellular accumulation of free HBxAg and increasing severity of liver disease, suggesting that at least some of these interactions may be relevant to pathogenesis. Further work needs to be carried out to understand the genes, the differential expression of which, as a consequence of

these interactions, plays a role in the pathogenesis of HCC.

Analysis of WHV DNA integration patterns showed that in chronically infected livers the integrated WHV DNA was often co-linear with that of virus DNA, whereas in HCC nodules the WHV DNA was often partially deleted and rearranged at the integration site.[21,22] In addition, the rearrangement of integrated HBV DNA in transgenic mice over time,[40] combined with the frequent loss of heterozygosity that accompanies the development and progression of HCC,[30] suggests that chronic hepadnavirus infections are associated with a considerable amount of genetic instability. The finding that HBxAg binds to and presumably inactivates an ultraviolet-light-induced DNA damage binding protein, which seems to be important for the recognition step of the excision repair pathway,[85] suggests that HBxAg may promote genetic instability in this way. Independent work has also shown that HBxAg disrupts the functional integrity of the p53-ERCC3 complex *in vitro*.[72] Since the latter complex is important for transcription-coupled repair, its inactivation may also contribute to the accumulation and propagation of mutations relevant to hepatocarcinogenesis. Although these results are provocative, it is not known whether these mechanisms are operative *in vivo*.

Additional evidence that HBxAg contributes to hepatocarcinogenesis is the appearance of altered foci, adenomas, and HCC in X-transgenic mice with persistently high levels of HBxAg expression.[48–50] These observations have not been reproducible in other X-transgenic mice,[86] but the levels and duration of HBxAg expression seemed to be much less in the latter studies. The striking observation that the X-transgenic mice that develop HCC do so in a background in which there is no hepatocellular turnover and no regeneration may be due to the fact that such mice have X gene sequences integrated into every cell, and that this may short circuit the need for hepatocellular turnover in promoting virus integration. These observations, combined with those in which HBxAg transforms only tissue culture cells that have already been through most of the steps required for transformation,[66,67,79] supports the idea that HBxAg only contributes a few steps in multistep hepatocarcinogenesis. The fact that HBxAg stimulates DNA synthesis when transiently introduced into fibroblast and liver cell lines[70,87] also suggests that to do so, HBxAg needs to overcome natural negative growth-regulatory pathways in the cell. This seems to occur when there are transiently high levels of HBxAg in tissue culture cells and when there are persistently high levels of HBxAg in the livers of chronically infected people and transgenic mice. Furthermore, the observation that HBxAg overcomes p53-mediated apoptosis in fibroblasts,[76] and that HBxAg stimulates the NF-κB[88–91] and Ras-Raf-mitogen-activated protein kinase[68–70,92] signal transduction pathways important to the controlled regulation of cell growth, may give cells a survival advantage over the 20 to 50 years it takes to develop HCC. In this context, sustained and increasingly elevated levels of intracellular HBxAg[25,47] would be necessary but not sufficient for hepatocellular transformation. Hence, HBxAg may be a prognostic marker associated with the development of HCC.[93]

HBxAg Functions Associated with Hepatocarcinogenesis

The most well-characterized property of HBxAg is its ability to mediate *trans*-activation of many cellular and viral promoters.[52] Among the natural targets of HBxAg *trans*-activation are the virus genes themselves, in that HBxAg function is important for maintaining wild-type levels of virus gene expression and replication both *in vitro* and *in vivo*.[55–58] HBxAg stimulation of virus gene expression may elicit antiviral immune responses capable of removing infected cells on one hand and providing regenerative stimulus in the liver on the other. Sustained, high levels of viremia may increase the number of infected hepatocytes, thereby increasing the magnitude of the cellular inflammatory responses (Figure 1). If true, then persistent HBxAg expression may accelerate the pathogenesis of HCC by encouraging persistent hepatocellular regeneration associated with bouts of chronic liver disease. There is experimental support for this view.[93]

The finding that HBxAg may be a protein kinase with apparently autophosphorylating activity,[94] and that it readily becomes phosphorylated in human hepatoma cells,[95] implies that phosphorylation may contribute to carcinogenesis. Although the targets for HBxAg phosphorylation are not known, the phosphorylation of tumor suppressor gene products and cell cycle proteins are known to alter their activities in the control of cell growth.[96] More recent work, however, has failed to demonstrate that HBxAg is a protein kinase, although HBxAg had ATPase and deoxy-ATPase activities *in vitro*.[97] These activities may be coupled to HBxAg-mediated *trans*-activation, since ATP hydrolysis is known to facilitate unwinding of the DNA template at the site of transcription initiation[98] and to activate a preinitiation complex in transcription through the phosphorylation of RNA

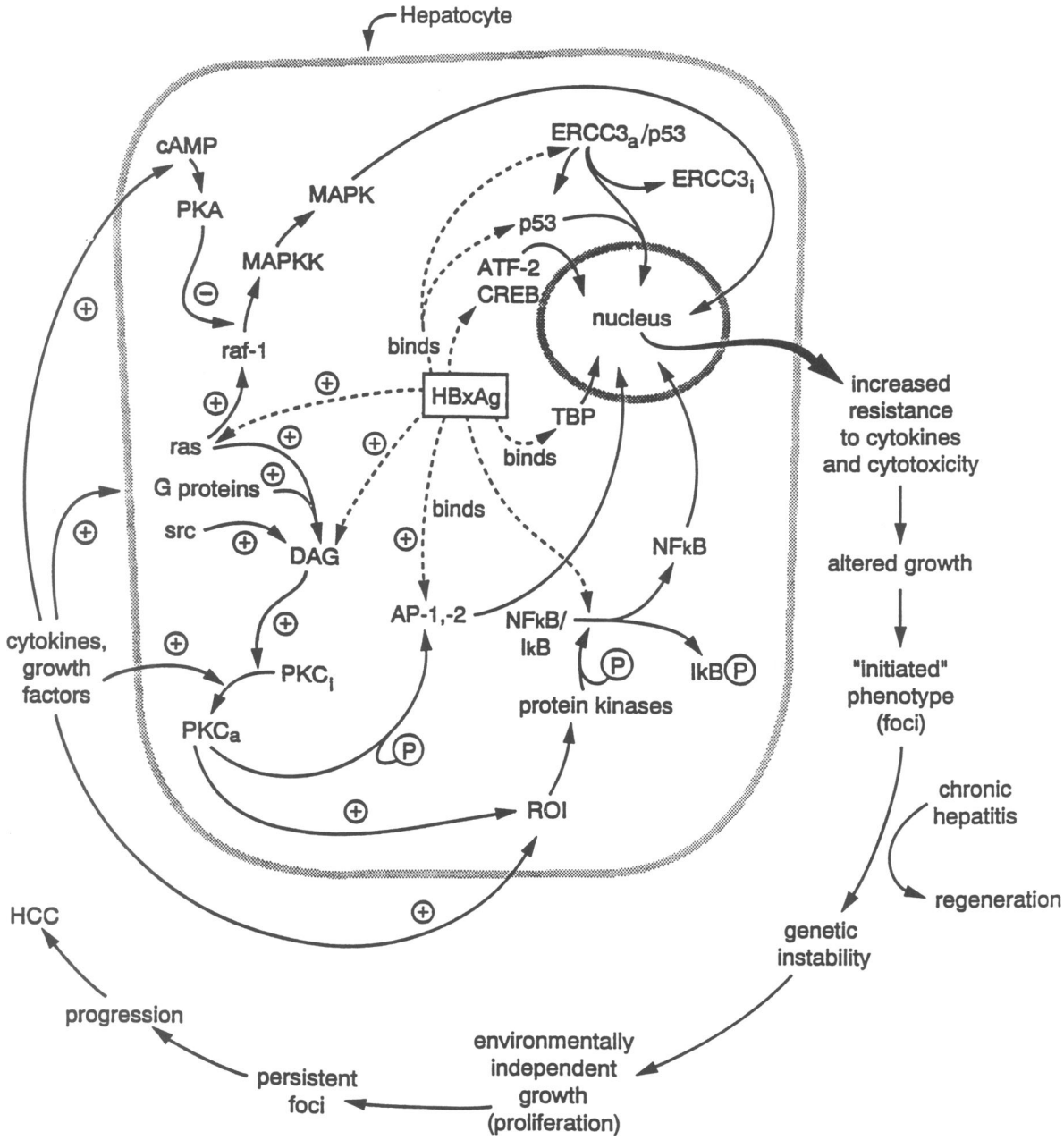

Figure 3. *Signal transduction pathways, the function of which may be altered by HBxAg. HBxAg may up- or down-regulate these pathways* (dashed lines) *by directly binding one or more critical components of the pathways, such as the TATA-binding protein (TBP), activating transcription factor-2, cAMP response element-binding protein (CREB), p53, or AP-1 and -2. Alternatively, or in addition, HBxAg may indirectly stimulate the* ras *and/or NF-κB signal transduction pathways by mechanisms that are not yet clear. In any event, the altered patterns of gene expression may result in an increased resistance of such cells to apoptotic signals provided by the immune system in the form of cytotoxic cytokines. The survival advantage of HBxAg-positive cells would then be a prerequisite for additional changes, also mediated by HBxAg, which would promote the accumulation of mutations which contribute to the development of HCC.*

polymerase II.[99] Given that HBxAg *trans*-activation seems to be mediated through phosphorylation-dependent signal transduction pathways (Figure 3), if HBxAg stimulates the activity of one or more key protein kinases in these pathways or inhibits the activity of corresponding phosphatases, signal transduction may be altered in HBxAg-positive compared with negative cells. Since alterations in host gene expression are often mediated by changes in the activity of signal transduction pathways, the putative pleiotrophic effects of HBxAg in carcinogenesis may be explained, in part, by the inappropriate activation of these pathways. For example, in F9 teratocarcinoma cells, which lack the protein kinase

C (PKC)-dependent transcription factor AP-1 (which consists of Jun and Fos), HBxAg *trans*-activation was dependent on the cotransfection of Jun and Fos expression plasmids (Figure 3).[88,89] The observation that HBxAg increased the binding of AP-1 to cognate DNA sequences in a human liver-derived cell line (CCL13), and that this binding was abolished by treatment of the cells with a PKC inhibitor, suggests that HBxAg *trans*-activation may depend on PKC.[88] HBxAg transfection also resulted in an increase in the endogenous PKC activator 1,2-diacylaglycerol (Figure 3). Since PKC and AP-1 function through NF-κB-dependent signal transduction pathways, as do Src and Ras, for example, HBxAg may inappropriately activate some of the same pathways regulated by cellular oncogene products. The fact that both AP-1 and NF-κB are normally stimulated by cytokines and growth factors is also consistent with their induction by HBxAg. Independent observations also suggest that HBxAg stimulates the activities of the AP-1- or AP-2-associated signal transduction pathways.[89] Hence, HBxAg has some features in common with growth-regulatory proteins. However, it remains to be seen whether any of these pathways are altered *in vivo*, and if they are, whether these alterations are relevant to the development of HCC.

HBxAg has also been found to mediate activation of NF-κB in PKC-independent pathways.[90,91] In PKC-associated signal transduction, NF-κB exists complexed to its inhibitor (IκB) in the cytoplasm, in which it remains inactive. When IκB becomes phosphorylated, active NF-κB is released for transport and functions in the nucleus. The recent demonstration that activated NF-κB prevents apoptosis,[100,101] that NF-κB is required for hepatocellular viability and growth during embryogenesis,[102] and that HBxAg stimulates NF-κB[88–91] suggests that X antigen may promote the survival of both infected and mutated cells during repeated bouts of chronic liver disease. This may contribute importantly to the development of HCC. Furthermore, in PKC-independent pathways, IκB becomes phosphorylated by kinases (other than PKC) stimulated by the generation of reactive oxygen intermediates (ROIs)[103,104] resulting from the action of some cytokines. If HBxAg is a protein kinase or is physically bound to a cellular protein kinase (which may result in HBxAg phosphorylation), it may also activate NF-κB-dependent pathways by stimulating phosphorylation of IκB. The finding that HBxAg *trans*-activation of NF-κB-controlled reporter genes is inhibited by antioxidants[105] suggests that HBxAg may promote NF-κB genes in the presence of ROI in infected cells. Independent work, showing that the CAH in hepatitis-B-surface-antigen-overproducing transgenic mice destined to develop HCC is associated with extensive oxidative DNA damage,[106] also suggests a role for ROIs in pathogenesis. The central role of free radicals in the initiation and progression of multistage carcinogenesis[107,108] may be one of the ways whereby HBxAg contributes to this process. The combination of elevated ROIs and increased HBxAg expression in the liver may also provide a partial explanation for the increased susceptibility of HBV-transgenic mice to chemical carcinogen-induced tumors.[109] In this context, the detection of sustained aflatoxin consumption in regions of the world with a high frequency of both chronic carriers and HCC[110,111] also implies synergy between HBV and this potent hepatocarcinogen. However, the relationship among elevated ROIs, chronic HBV infection, and aflatoxin in the pathogenesis of HCC remains to be clarified.

Recent work has also suggested that HBxAg shares limited homology with Kunitz-type serine protease inhibitors,[112] the latter of which play significant roles in carcinogenesis by stimulating cellular growth.[113] Mutation of the region in HBxAg having homology with the "Kunitz domain" of these protease inhibitors also abolished its *trans*-activation function, suggesting that HBxAg may bring about *trans*-activation by inhibiting the proteolytic cleavage and effectively increasing the half-lives of selected transcriptional proteins. The association of HBxAg with the proteasome is consistent with these findings.[114] More recently, HBxAg has been shown to bind and inhibit the activity of the hepatic serine protease tryptase TL_2 without being degraded, implying that HBxAg may act as a protease inhibitor.[115] Inhibition of the related tryptase TL_1 was also observed.[115] Given that the natural substrate(s) and inhibitor(s) of these enzymes have not been identified, the significance of the HBxAg-tryptase TL relationship to HCC remains to be firmly established.

The possibility that HBxAg resembles a protease inhibitor may have other ramifications relating to the development of HCC. For example, it is well known that the α_1-antitrypsin deficiency in humans[116] and in transgenic mice,[117] which often results in the appearance of HCC, is associated with the abnormal accumulation of the antiprotease in the liver, with a corresponding deficiency in the blood. This accumulation is toxic, in that it results in the loss of hepatocytes, followed by regeneration, and the loss of the transgene expression (in mice) among surviving cells. Cell loss and regeneration are also prominent in transgenic mice that develop HCC associated with the overexpression of transformation growth factor α,[118] Simian virus 40 T antigen,[119] or the hepatitis B

surface antigen.[28] If HBxAg acts as an antiprotease in natural infection, it may stimulate both cell loss and regeneration in a dose-dependent manner, independent of antiviral immune responses, thereby contributing importantly to the pathogenesis of HCC.

The context of HBxAg expression at different times during infection, which determine its subcellular localization, binding partners, and function(s), is likely to critically determine how and when HBxAg contributes to the development of HCC. One hint may lie in the observation that early on in chronic infection the liver is composed largely of hepatocytes that are sensitive to HBV infection and support virus replication (ie, quiescent and fully differentiated cells or S cells), whereas late in chronic infection, the liver is composed largely of hepatocytes that are resistent to HBV infection and are largely nonpermissive for virus replication (ie, regenerating, less-differentiated R cells).[120,121] Since HBV replication occurs best in quiescent and fully differentiated liver cells and cell lines,[74,75] the shift in the hepatocellular environment in which HBxAg is expressed will slowly change during the course of chronic infection. If these changes are reflected in different HBxAg-binding partners in S compared with R cells, the characteristics of HBxAg may vary greatly in these different circumstances. Although the existence of R and S cells is not certain at the present time, systems are available to test this hypothesis experimentally.

The Relationship between HBxAg and p53 in the Pathogenesis of Chronic HBV Infection and the Development of HCC

There is increasing evidence that tumor suppressor proteins, such as p53, negatively regulate cell growth, thereby acting to prevent the uncontrolled cell proliferation characteristic of tumors.[122,123] The centrality of p53 to the maintenance of cellular genome integrity is highlighted by observations that p53 expression is induced in cells that sustain radiation-induced DNA damage[124,125] and that this induction prevents cell division until DNA repair is completed. When p53 function is lacking, cells with damaged DNA continue to replicate and usually die or, rarely, transform. The importance of p53 is further underscored by the fact that point mutations, which inactivate this protein, have been found in many tumor types.[126] Among DNA tumor viruses, transformation involves the appearance of virus proteins, which bind to and inactivate p53, and in some cases, other tumor suppressor products as well.[127] p53 knockout mice have an increased risk for the development of many types of cancers.[128] Hence, functional inactivation of p53 is a common feature of carcinogenesis.

The integrity of p53 is also important to the mechanism(s) operative in HBV-associated HCC. The fact that HBV DNA integration into the human genome is common, that it occurs in most chromosomes examined,[33] that hepatocellular regeneration during bouts of chronic hepatitis promotes integration, and that integration is associated with chromosomal instability,[40,129] suggests that chronic HBV infection is associated with prolonged periods of DNA damage, which may require the function of p53 to affect DNA repair prior to mitosis. Hence, the integrity of p53 seems to be central to limiting DNA damage resulting from HBV integration events and the subsequent development of HCC. ROIs may also contribute to DNA damage in patients with CAH. In this context, HBxAg was found to increase the radiation sensitivity of the human hepatoma line HepG2 to X irradiation, implying that HBxAg may inactivate p53.[130] An association between HBxAg and p53 was observed in human livers from carriers with HCC by co-immunoprecipitation of both these polypeptides with anti-HBx or anti-p53 and confirmed with similarly designed experiments using *in vitro*-translated products.[71–73] Further work showed that HBxAg relieved p53 suppression of transcription of promoters lacking a p53-responsive element[131,132] and stimulated expression of promoters having one or more p53-responsive elements.[54] The latter result suggests that HBxAg *trans*-activation of p53-responsive genes may be mediated by HBxAg-p53 complex formation. Further work showed that p53 suppression of individual HBV promoters was relieved by HBxAg,[133] but that when the virus promoters were tested again in their natural context within the intact virus genome, HBxAg and p53 acted synergistically in the stimulation of HBV replication. Further analysis showed that this phenomenon was associated with the presence of a p53 binding site within HBV DNA (X. L. Duan, M. Zhu, M. Feitelson, I. Osak, B. Sun, J. Guo, and R. Pomerantz, submitted for publication). The finding that HBxAg inhibits the binding of p53 to its responsive element *in vitro*,[72] however, seems to be in contrast to the results presented above, but this discrepancy may be due to the HBxAg:p53 ratios used in each case. It is proposed that at low HBxAg:p53 ratios, HBxAg binds p53 at its responsive element, further stimulating transcription, whereas at high ratios, HBxAg displaces p53 from its responsive element (Figure 4). Low HBxAg:p53 ratios seem to predominate early in infection, when the small amount of free HBxAg in the cell would act to stim-

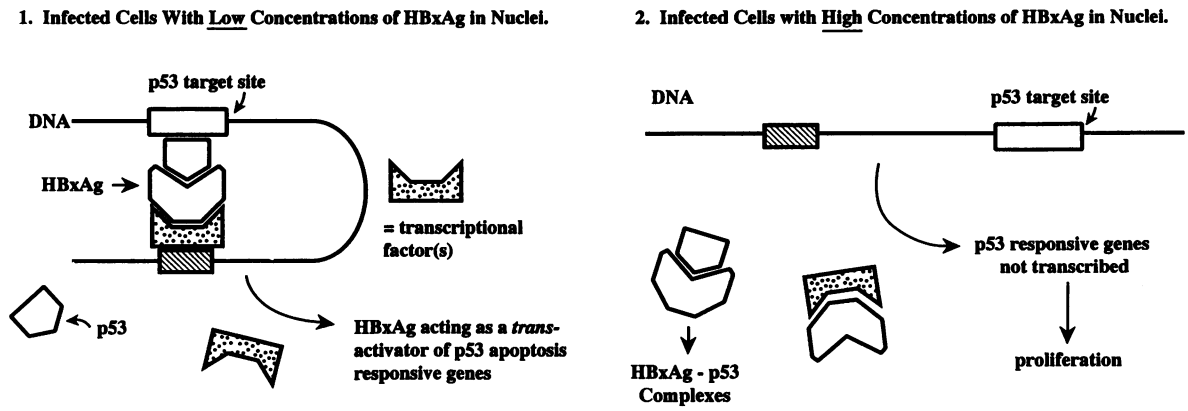

Figure 4. *Speculative model showing that the functions of HBxAg may vary, depending on the amount of HBxAg, which increases during chronic infection. The binding of HBxAg to p53 is presented as an example, but the rationale could be extended to other transcription factors that physically and/or functionally interact. At low HBxAg concentrations, the binding of HBxAg to p53 stimulates the expression of p53 target genes. With respect to the virus, this would result in increased virus replication. With regard to the infected cell, this may result in G_1 arrest. Since quiescent cells fully support virus replication, this may play a role in the persistence of virus replication and maintenance of the chronic carrier state. As viral integration accumulates over time, and intracellular levels of HBxAg increase, it is postulated that the functional complexes between HBxAg and transcription factors begin to break down, resulting in phenotypic changes among HBxAg-positive hepatocytes that are important for subsequent changes to occur in the pathogenesis of HCC.*

ulate HBV gene expression and replication in a p53-dependent fashion. High HBxAg:p53 ratios seem to predominate late in infection when HBxAg is made in relatively large amounts from integrated templates and accumulates within infected cells. Additional work has shown that HBxAg does not degrade p53 *in vitro*,[131] as does human papilloma virus 16 E6, suggesting that the functional inactivation of p53 may involve conformational changes in p53 on complex formation or the displacement of p53 from some of its usual substrates by HBxAg. These p53 substrates may include the homolog of the oncogene product mouse double minute 2,[134,135] the deregulated expression of which is associated with some tumors,[136] the growth arrest DNA damage complex promoter,[137] TATA-binding protein,[138] heat shock protein 70 and its corresponding promoter,[139,140] and other genes important in the regulation of cell growth and differentiation.[134] Hence, HBxAg-p53 complex formation may alter genetic stability and cell cycle control, both of which are central to multistep carcinogenesis.

HBxAg-p53 complexes have been found in the HCCs that develop in X-transgenic mice.[50] Co-staining of HBxAg and p53 in the same cells from altered foci, adenomas, and HCC nodules, further suggests a close relationship between these proteins. In these mice, HBxAg seems to inactivate p53 by sequestration of the latter in the cytoplasm. Suppression of HBxAg expression by short-term interferon treatment resulted in the loss of cytoplasmic p53 and reappearance of p53 in the nuclei of hepatocytes. Sequence analysis of p53 exons 5 through 8 in these mice did not reveal any evidence of inactivating point mutations, suggesting that the inactivation of p53 in HCC nodules was associated with HBxAg-p53 complex formation. In human HCC, elevated levels of p53 were observed in the nuclei of infected cells, and in some cases, nuclear HBxAg was also observed in the same cells.[141] Exon sequencing showed that the p53 staining was associated with wild-type p53 in most cases. The results are compatible with the conclusion that HBxAg stabilizes p53 in either a wild-type or mutant conformation. Although more work is required to test this hypothesis, the normal turnover of p53 by ubiquitination in the proteasome[142] may be altered if HBxAg binds to p53 and acts as a protease inhibitor. In this context, the recent demonstration that HBxAg binds to a subunit of the proteasome complex, and that such binding correlates with the ability of HBxAg to carry out *trans*-activation,[114] suggests that the proteasome may be a functional target for HBxAg in infected cells. Altered proteasome function would not only stabilize molecules such as p53 and the cyclin-dependent kinase inhibitor, $p21^{WAF}$, but may also inhibit the degradation of oncogene products and cellular *trans*-activating proteins such as c-Jun, c-Fos, and NF-κB, which are important in the regulation of cell growth.[143,144] In this light, it may be more than just

coincidence that HBxAg *trans*-activation is associated with the increased activity of c-Jun, c-Fos,[69,145] and NF-κB.[146] There also exists the possibility that the improper degradation of viral proteins in the proteasome will alter their ability to interact with the appropriate human leukocyte antigen molecules,[147] which may contribute importantly to the escape of virus infected cells from immune recognition and elimination.

There is also a correlation between p53 inactivation and defects in DNA repair,[137,148] which together may promote genomic instability. As mentioned earlier, the normal binding of p53 to ERCC3, the latter of which is a basic transcription factor involved in transcription-coupled DNA repair,[149,150] seems to be disrupted by HBxAg.[72] The likelihood that p53 suppresses transcription, in part, by inhibiting the activity of DNA helicases,[151] and that ERCC3 has intrinsic helicase activity,[149] suggests that p53 inactivation by HBxAg would result in unscheduled and unregulated transcription (Figure 3). Replication and transcription of damaged cellular DNA would result in the propagation of mutations that contribute to multistep carcinogenesis. Emerging evidence that mutations in cellular DNA are related to the slow repair of ultraviolet-light-associated DNA damage in skin cancer,[148] and that mutations within DNA repair enzymes are associated with the development of the hereditary form of colon cancer,[152] further underscores the importance of the fidelity of DNA repair to carcinogenesis. The fact that the rodent ERCC3 product corresponds to the human nucleotide excision repair gene *XPBC*, which in mutant form is associated with xeroderma pigmentosum,[150] is consistent with the finding that HBxAg increases the radiation sensitivity of HepG2 cells.[130] On the molecular level, HBxAg inactivation of p53 through complex formation and the possible impairment of ERCC3, then, may contribute to other chromosomal alterations reported to be associated with HCC.[30,31] The accumulation of chromosomal alterations during tumor progression, including mutations in the *p53* gene itself,[153] suggest that HBxAg/p53 complexes are rate limiting early in tumor formation, whereas the appearance of chromosomal aberrations become dominant later on. However, direct evidence of the inactivation of one or more DNA repair enzymes in infected livers remains to be established.

HBxAg and Insulin-Like Growth Factor II

The finding that insulin-like growth factor II (IGF-II) expression, which is normally expressed only in fetal liver,[154] is elevated in both human[155–157] and woodchuck[158,159] HCCs, suggests that this enhanced expression may be an important feature of hepatocarcinogenesis. Elevated IGF-II expression has also been detected in premalignant proliferative nodules in both human and woodchuck HCCs,[156,157,159] suggesting that its reactivation is an early step in the development of this tumor type. The elevation of IGF-II expression in HCCs from HBV-infected but not uninfected patients suggests that HBV may stimulate IGF-II expression.[156] Further work demonstrated a correlation between IGF-II and HBxAg expression by immunohistochemistry,[160] suggesting that IGF-II may be a natural target of HBxAg-mediated *trans*-activation. However, the inverse correlation between IGF-II and WHV RNA levels in liver and tumors[158] suggests that IGF-II can be made independent of viral replication. In human hepatoma cell lines, IGF-II was expressed strongly in growing cells but was undetectable in confluent cultures,[160] suggesting that it is associated with cell proliferation, whereas viral replication was not.[75] The more recent finding that HBxAg stimulates the expression of the IGF-I receptor in human HCC cell lines,[161] which binds both IGF-I and IGF-II, suggests that HBxAg may set up an autocrine loop that enhances cell growth. Independent work strongly suggests that the IGF-I receptor is required for the establishment and maintenance of the transformed phenotype, both *in vivo* and *in vitro*, in many cell types.[162] If IGF-I receptor overexpression also occurs in the liver, it may contribute significantly to the survival and growth of HBxAg-positive cells and to the development of tumors. In the context of WHV transformation, the increased expression of N-*myc* in liver-derived cell lines increases the propensity of these lines to undergo apoptosis in the absence of serum. Addition of IGF-I, however, blocked apoptosis and stimulated cell growth in serum-free medium,[163,164] further suggesting the potential importance of this growth factor to hepatocarcinogenesis.

Conclusions

The likely pleiomorphic functions of HBxAg may distribute themselves among multiple pathways in the development of HCC. Alterations in any one pathway may be necessary but not sufficient for hepatocellular transformation. Although many pathways may be involved, as indicated above, those that are relevant to the pathogenesis of HCC need to be firmly identified. Presently, it is not known whether any of the HBxAg-associated functions are operative in the de-

velopment of HCC. HBxAg-p53 complex formation seems to be an important step in viral hepatocarcinogenesis, by analogy to other DNA tumor viruses, but the significance of these complexes to transformation requires further characterization. Likewise, the role of IGF-II and IGF-I receptor expression in hepatocarcinogenesis merits closer examination. Cellular targets that are altered during transformation can be identified, in part, by asking whether selected biochemical pathways are stimulated or depressed in preneoplastic or neoplastic tissues from woodchucks or patients and by developing additional experimental systems that closely mimic the disease process and biochemical alterations likely to be affected in human disease. In this way, research will be able to move from the realm of the possible to the realm of the relevant and, in doing so, to provide the appropriate systems for therapeutic intervention.

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