Tumorigenesis and Neoplastic Progression

Separate Origins of Hepatitis B Virus Surface Antigen-Negative Foci and Hepatocellular Carcinomas in Transgenic HBsAg (*alb/psx*) Mice

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We have examined the development and transgene expression in liver lesions of transgenic mice bearing the hepatitis B surface antigen (HBsAg) gene of hepatitis B virus under the control of the albumin promoter (*alb/psx*) to study liver regeneration and hepatocellular carcinoma (HCC) associated with hepatitis B virus infection. Storage of the HBsAg in the endoplasmic reticulum precedes loss of liver cells and regenerative hyperplastic nodules that do not express HBsAg. Histological analysis indicated that HBsAg-negative foci and nodules arose from liver progenitor cells in the portal zone and lacked mRNA expression. Genomic DNA from eight of nine HBsAg-negative laser capture-excised liver foci showed loss of part of the *alb/psx* gene, whereas no loss of the actin gene was observed. The *alb/psx* DNA was intact in adjacent HBsAg-positive tissue. Sequencing of polymerase chain reaction products suggested that alterations in the HBsAg transgene in HBsAg-negative foci occurred via large-scale deletions as opposed to single-site mutations. Southern blot analysis of HCC from 2-year-old transgenic HBsAg mice, however, revealed an intact *alb/psx* gene. Thus, HBsAg-negative progenitor cells with deletions in the transgene appear to be responsible for compensatory regeneration of the liver, whereas HCCs arise from clonal expansion of hepatocytes with intact *alb/psx* transgenes. (*Am J Pathol* 2006, 169:223–232; DOI: 10.2353/ajpath.2006.051284)

United States over the last two decades, from 1.4 in 100,000 to 2.4 in 100,000.² Because of difficulty in diagnosing early HCC, it has a high mortality rate compared with most other cancers.¹ HCC is the predominant cause of cancer mortality in sub-Saharan Africa and southern China.^{3–6} It causes 65 to 75% of all cancer deaths in males and 30 to 55% of cancer deaths in females in Mozambique and in some provinces of southern China.^{6–16} Epidemiological studies have identified infection with hepatitis B virus (HBV) and contamination of peanuts or grain with aflatoxin (AFB1) as major and possibly synergistic risk factors in these areas.^{4–17} Individuals with chronic HBV infection have a 200-fold greater risk of developing HCC than do age-matched noninfected controls.¹

Multiple factors appear to contribute to HBV infection-induced pathogenesis of liver cancer. Chronic infection and production of cytokines play roles in the development of fibrosis and in liver cell proliferation.^{15–21} Disruption or promotion of genes associated with the cell cycle, growth, and oncogenic pathways that are present in close proximity to the site of HBV integration have been implicated in transformation and cancer development.^{21–26} Similarly, HBV-encoded proteins can contribute to the pathology of the cells of the liver.^{21,27–31}

Various aspects of HBV biology and molecular pathogenesis can be addressed through the development of transgenic mouse models with organ-specific expression of viral genes. In the transgenic mouse lineage 50-4 or Tg(Alb-1HBV)Bri44 developed by Chisari et al,³² the HBV *BamHI/BglII* gene fragment is linked to the albumin promoter directing expression in liver cells. This hepatitis B surface antigen (HBsAg) transgenic mouse lineage with a C57BL/6 background expresses the HBsAg polypeptide in the cytoplasm of hepatocytes but does not express the X-protein.³³ The hepatocytes of the mice of this line do not secrete the large HBsAg but rather store it in

Hepatocellular carcinoma (HCC) accounts for 1% of new cancers detected per year and more than 2% of cancer deaths in the United States.¹ There has been a statistically significant increase in the incidence of HCCs in the

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the endoplasmic reticulum, resulting in liver dysplasia at about 3 months of age.³⁴ This is followed by severe prolonged hepatocellular injury and hepatocyte death, increased generation of reactive oxygen species, regenerative hyperplasia beginning at about 6 months of age, and aneuploidy, oval-cell proliferation, nodule formation, and eventual development of HCC at 15 to 18 months of age in males.^{32–38} One of the characteristic observations in these mice has been the progressive loss of HBV-specific antigen in the liver corresponding to the appearance of HBsAg-negative microscopic nodules. Later, HCCs develop, and these are also HBsAg negative.^{33,34} Thus, there appears to be a relationship between HBsAg nodular regeneration and HCC development.

Southern blot analysis of genomic DNA probed with full-length HBV DNA probe showed identical HBV-specific fragments in HCC from all of the 16 mice examined as well as in the tail samples from these mice.³³ The authors suggested that despite the apparent integrity of the integrated transgene, the cells of HCCs are transcriptionally altered. Here, we use immunohistological analysis of HBsAg expression to study the cellular origins of HBsAg-negative foci and nodules. In addition, we examine the expression and gene structure of HBsAg in pre-malignant liver foci as a means to understand the loss of HBsAg protein expression, a step believed to precede development of HCC in this model.

Materials and Methods

Transgenic HBsAg Mice

Transgenic HBsAg mice were obtained from Dr. Francis Chisari.^{32,33} This transgenic strain contains an HBV *Bgl*Ila DNA fragment coding for the entire HBsAg antigen under the transcriptional control of the mouse albumin gene promoter directing expression to liver cells (*alb/psx*). The inserted *alb/psx* gene segment also includes sequences coding for the X-protein of HBV, but the X-protein is not expressed in this transgenic lineage. The official designation for these mice is Tg(Alb-1HBV)Bri44, and they are more commonly referred to as lineage 50-4.^{33,35} These mice were housed and maintained at the Wadsworth Center Animal Facility under institutionally approved conditions.

Immunohistochemistry

Tissue blocks from the liver were fixed in buffered formalin for 8 hours, embedded in paraffin, and cut into 6- μ m-thick sections according to previously described conditions.^{35,38} Sections were deparaffinized and rehydrated using standard methods including xylene and decreasing concentrations of ethanol. Before staining, antigen retrieval was performed using 0.1% trypsin in 0.05 mol/L Tris-HCl, pH 7.8, and 0.1% CaCl₂ buffer at 37°C for 20 minutes. After antigen retrieval, sections were rinsed in water for a total of 10 minutes and incubated in a 1:50 dilution of 30% H₂O₂ in 100% methanol. Sections were

then rinsed in water for a total of 10 minutes and equilibrated in 1× Tris-buffered saline.

Double labeling for HBsAg and cytokeratin was performed sequentially. HBsAg was labeled using goat anti-HBsAg monoclonal antibody (1:1000 dilution; Dako-cytomation, Carpinteria, CA) followed by horseradish peroxidase-labeled donkey anti-goat IgG (1:500; Jackson ImmunoResearch, West Grove, PA). Cytokeratin labeling of bile ducts was performed as described previously³⁹ using rabbit anti-cytokeratin wide spectrum (1:100; Dakocytomation) and alkaline phosphatase-labeled anti-rabbit IgG (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). Pan-cytokeratin (Pan-Ck) labeling was developed using NBT/BCIP, and HBV staining was developed using NovaRed (Vector Laboratories, Burlingame, CA).

In Situ Hybridization

To determine the expression of the HBsAg in the nodules, we embedded liver tissue from 9-month-old HBV-transgenic mice in OCT compound (Sakura Finetek, Inc., Torrance, CA) and kept it frozen at –80°C until cryo-dissection. *In situ* hybridization for HBsAg mRNA in liver sections was performed using digoxigenin-labeled RNA probes prepared in both the sense and antisense direction according to the instructions in the DIG RNA Labeling kit supplied by the manufacturer (Roche, Indianapolis, IN). The DNA template for HBsAg was plasmid pRc/CMV-HBs(S) containing a 2-kb *Bss*III-*Kpn*I fragment of HBs(S).

Laser Capture Microscopy

Laser-assisted microdissection was performed on formalin-fixed, paraffin-embedded 6- to 8- μ m liver sections using the PALM Robot-Microbeam laser microdissection system (PALM Microlaser Technologies, GmbH, Bernried, Germany). The system is based on a sharply focused laser beam used to cut a circle (approximately 300 to 600 μ m in diameter) around a selected area of cells. Sections of liver nodules negative for HBsAg and extra-nodular areas positive for HBsAg were laser-dissected and collected separately into 0.5-ml polymerase chain reaction (PCR) tubes containing 30 μ l of catapult/digestion buffer as instructed by PALM Microlaser Technologies (1 μ mol/L ethylenediamine tetraacetic acid [pH 8.0], 20 μ mol/L Tris [pH 8.0], 0.5% Igepal CA-630 [Sigma, St. Louis, MO], and 0.2 μ g of proteinase K). Samples were digested at 55°C for 18 hours and then heat-inactivated for 5 minutes at 95°C. Aliquots of these were used in the PCR reaction mixture together with the appropriate PCR primers as described below.

PCR

Three sets of overlapping genomic PCR primers and corresponding nested primers obtained from Integrated DNA Technologies, Inc. (Coralville, IA) were used to amplify the entire length of the HBsAg transgene (see Results for further description of probes). The microdis-

sected tissue samples obtained as described above from HBsAg-negative or -positive pools were tested separately in the PCR reaction with PCR primers for β -actin and three sets (I, II, and III) of HBV genomic primers, followed by reamplification of 2 μ l of PCR-reaction mixture product with HBV-nested primers. PCR was performed in a Perkin Elmer 480 Thermal Cycler (Perkin Elmer, Boston, MA) for 45 cycles with successive denaturation, annealing, and extension stages set at 95, 55, and 72°C, respectively. The conditions for the two PCR reactions were identical.

Southern Blot Analysis

Fresh tissues obtained from visible liver tumors, kidney tissue, and nontumor liver tissues from 2-year-old transgenic mice were processed for DNA extraction using the GenElute Mammalian Genomic DNA Miniprep kit (Sigma). Approximately 20 μ g of DNA was digested with *Eco*RI restriction enzyme, and DNA fragments were separated on 1% gel agarose and transferred to Gene Screen membrane (NEN Life Sciences, Boston, MA). These blots were then probed with PCR products labeled with Megaprime DNA (Amersham Biosciences, Piscataway, NJ) from HBV-gene template using a standard technique as described.^{40,41} The primer set II product described above was used as a template for labeling HBV.

Sequencing

After electrophoresis, PCR product amplicons were excised and DNA purified using the Roche gel excision kit according to the manufacturer's instructions. Extracted DNA was then cycle sequenced by the Wadsworth Center Molecular Genetics Core Facility under the direction of Dr. Matthew Shudt.

Results

Generation of HBsAg-Negative Foci and Nodules in Transgenic HBsAg Mice

The sequence of pathological changes in HBsAg transgenic mice is as follows: 1) storage of HBsAg in the endoplasmic reticulum, leading to large swollen hepatocytes beginning at birth; 2) hepatocyte injury and dysplasia beginning at about 3 months; 3) cell death, development of hyperplastic nodules, and oval cell proliferation, beginning about 6 months; and 4) hepatocellular carcinoma beginning about 15 months in males and death by 18 months. A similar sequence occurs in females, but in many animals there is regeneration of the liver with HBsAg negative cells and survival for over 2 years without development of HCC.^{38,42} For the present study, we targeted events occurring after the initiation of injury and regenerative hyperplasia in males but before the formation of visible HCC. Liver sections from HBV transgenic mice at various ages were prepared and analyzed for the

expression of HBsAg protein. At 5 months of age, essentially all hepatocytes of transgenic HBsAg mice contain HBsAg, but other than enlarged hepatocytes, the structure of the liver is not different from that of nontransgenic mice (Figure 1, A and B). By 7 to 8 months, foci of HBsAg-negative cells are seen (Figure 1C). There is a progressive increase in cells not expressing HBsAg, first in small foci, then in nodules (Figure 1, D and E; 12 months), and finally in multiple nodules and microscopic HCCs (Figure 1F; 15 months). Essentially all HCCs in this model are HBsAg negative.^{34,35} Oval cell proliferation is seen after 8 to 10 months (not shown). These results are consistent with previously published reports and suggest that HBsAg-negative nodules arise from HBsAg-negative foci and that HCCs arise from HBsAg-negative nodules.^{34,35,43} Sections showing early HBsAg foci and nodules, such as that shown in Figure 1, C–E, suggest that early foci are closely associated with ducts in the portal zone.

To clarify a possible relationship between early foci and ductal proliferation, we stained sections with antibodies to cytokeratin to reveal possible duct formation as part of the process of HBsAg focus formation. Figure 1, G–I, shows examples of this staining in normal mice and transgenic mice at 2.5 months and in transgenic mice at 9 months. At 2.5 months (Figure 1H), small ducts were limited to the portal zone in both normal nontransgenic mice (not shown) and in transgenic mice. By 9 months, there was a progressive increase in small ducts extending from the portal zone into the adjacent hepatic lobules in transgenic mice (Figure 1I), but the location of ducts in nontransgenic mice was the same as seen at 2.5 months (Figure 1G). To show the location of HBsAg foci, we cut 20 serial sections to trace the location of the foci in the liver. As shown in Figure 2, top panels, the HBsAg-negative focus in 9-month-old male transgenic mice can be followed from the liver lobule to the portal zone and then back to the lobule. This suggested that the HBsAg-negative foci have a close relationship with the portal zone and to biliary ductules. To examine the relationships between these ducts and HBsAg nodules more closely, we double-stained sections for Pan-CK and for HBsAg. Twenty serial sections were cut from each of five transgenic HBsAg mice at 8 months of age. Figure 2, middle panels, shows representative selected serial sections from one of these mice. The arrows in the middle panels point to a close association of the small HBsAg focus with Pan-CK-positive small ducts. A total of 76 HBsAg-negative foci were followed in this manner, and 78% of these could be shown to be adjacent to small biliary ductules. We chose livers from 8-month-old male mice for further analysis of HBsAg expression because at this age, especially in male mice, consistent and well-defined HBsAg nodules could be obtained.

HBsAg mRNA Expression Is Absent in the Liver Foci of Transgenic Mice

To determine whether the observed absence of HBsAg protein expression was due to a loss of protein antige-

Figure 1. A–F: HBsAg staining. A, normal liver; B–F, transgenic male mice (brown color). G–I, Pan-CK staining (black color). A, normal mouse liver, 15 months; original magnification, $\times 400$; B–F, transgenic HBsAg mice. B, 5 months; original magnification, $\times 400$; C, 7 months; D, original magnification, $\times 200$; E, original magnification, $\times 400$; F, 15 months; original magnification, $\times 20$. B: There is uniform accumulation of HBsAg and dysplasia in each hepatocyte at 5 months. C–E: By 7 months, foci of small HBsAg-negative hepatocytes next to ductules (arrow) appear. An HBsAg-negative microcarcinoma is shown in F next to an HBsAg nodule at 15 months. G, Normal male mouse, 9 months; original magnification, $\times 200$; H, transgenic male, 2.5 months; original magnification, $\times 200$; I, transgenic male, 9 months. Arrows in I point to Pan-CK-positive small ductules extending into hepatic plates. p, portal vein.

nicity, to a loss of translatable mRNA, or to a loss of total mRNA, we analyzed liver sections from 9-month-old HBV transgenic mice for the expression of HBsAg mRNA. This was performed by *in situ* hybridization using the complete HBsAg-region DNA as a probe. HBsAg mRNA expression was not observed in foci (Figure 2, bottom panels). Thus, the loss of HBsAg protein expression in liver foci is due to the loss of mRNA template.

Analysis of HBsAg Genomic DNA in Liver Foci

The loss of HBsAg mRNA expression suggested the possibility that chromosomal changes in the HBsAg transgene occurred, in turn preventing HBsAg transgene transcription. To address this possibility, we analyzed the genomic DNA in HBsAg-negative foci. Laser capture

microscopy was used to obtain small areas of tissue situated completely within foci, and the transgene was analyzed in these areas using PCR. The laser capture approach was important for two reasons. First, it ensured that tissue from one focus at a time was isolated. This avoids the potential heterogeneity that may occur in less accurate focus removal, during which combining of foci may occur. Second, the surrounding (nonfocal) tissue in which HBsAg mRNA expression was evident could be excluded. An approximately 300 to 600 μm diameter area could be routinely cut from a focus, clearly avoiding surrounding HBsAg-positive tissue.

We used PCR analysis of DNA extracted from individual foci to determine whether there was genomic loss of the HBsAg transgene. Genomic DNA was prepared as described in Materials and Methods. Several

Figure 2. Sequential sections of HBsAg-negative foci. **Top panel:** HBsAg staining, 8-month-old transgenic male mouse (original magnification, $\times 200$). **Middle panel:** Double staining for HBsAg (red) and Pan-CK (blue), 8-month-old transgenic male mouse. In the **top panel**, an HBsAg-negative focus can be followed from the liver lobule to the portal zone and then back to the lobule. In the **middle panel** are representative sections from 20 serial sections. The **arrows** point to small bile ductules associated with an HBsAg-negative focus. p, portal vein; n, HBsAg-negative focus. **Bottom panels:** mRNA expression in the liver foci of transgenic mice. Liver sections from 7-month-old HBV transgenic mice analyzed for HBsAg mRNA using *in situ* hybridization. **A**, mRNA (Green); **B**, nuclei (red, propidium iodide); **C**, double stained. Lack of HBsAg mRNA is observed in foci compared with surrounding (nonfocal) tissue.

Figure 3. PCR analysis. **A:** PCR primers used for amplification of the HBV transgene. Location of three PCR primer combinations with internal nested primer sites in the *alb/psx* transgene. For clarity, two panels are shown. **Top panel:** Overlapping PCR primers only. **Bottom panel:** Same as **top panel** but with internal nested PCR primer sites indicated. **B:** Specificity of PCR primers. Genomic DNA from positive control transgenic liver (+) and six nontransgenic mouse livers (S1 to S6) was extracted and PCR amplified using the primers described in the **top panel** of **A** (listed to the left of the figure), followed by re-amplification with the nested primers shown in the **bottom panel** of **A**. **Lane 1**, size markers. **Lane 2**, positive control (+). **Lane 3**, PCR performed in the absence of DNA template negative control (-). **Lanes S1 to S6**, nontransgenic liver samples. **C:** HBsAg-negative foci. Genomic DNA from positive control transgenic liver (+) and nine immunohistochemically HBsAg-negative individual nodules (**lanes F1 to F9**, **F1 to F3**, **F4 to F6**, and **F7 to F9** are from three different mice) was extracted and PCR amplified using the primers described in the **top panels** of **A** and then with the nested primers shown in the **bottom panel** of **A** (listed on the left). **Lane 1**, size markers. **Lane 2**, positive control (+). **Lane 3**, negative control (-). **Lanes F1 to F9**, HBsAg-negative foci samples. **D:** HBsAg-positive tissue surrounding the foci (HBsAg-negative foci). Genomic DNA from positive control transgenic liver (+) and six samples of immunohistochemically HBsAg-positive cells surrounding the foci (**lanes P1 to P6**) was extracted and PCR amplified using the primers described above (listed on the left). **Lane 1**, size markers. **Lane 2**, positive control (+). **Lane 3**, negative control (-). **Lanes P1 to P6**, HBsAg-positive tissue samples. P1 and P2 are from the same mouse as F1 to F3 in **C**; P3 and P4 are from the same mouse as F4 to F6; and P5 and P6 are from the same mouse as F7 to F9.

PCR primers combinations were chosen to generate overlapping amplicon product within the HBsAg transgene, as shown in Figure 3A.

To assess the specificity of these primers, we analyzed positive control transgenic liver and negative control nontransgenic liver samples. As expected, the transgene-positive control sample generated PCR product with all three overlapping HBsAg primers (Figure 3B, lane +), whereas nontransgenic liver did not show any PCR product with the same primers (Figure 3B, lanes S1–S6). These results confirmed the specificity of the primers and their utility in the analysis of tissue samples.

Genomic DNA from nine immunohistochemically HBsAg-negative foci was then analyzed (Figure 3C). Detection of PCR product from the β -actin gene was

observed in all foci. However, parallel analysis of the same focal DNA with HBsAg primers produced products with only some of the primer pairs and samples, indicating a loss of DNA. Specifically, three of nine samples exhibited a loss of DNA in the primer set 1 region, five of nine exhibited a loss of DNA in the primer set 2 region, and six of nine exhibited a loss of DNA in the primer set 3 region. Altogether, eight of nine foci lost at least some of the HBsAg gene. In contrast, DNA extracted from six regions surrounding the foci that were immunohistochemically positive for antibody to HBsAg contained DNA template to the complete set of HBsAg primers as evidenced by the production of PCR product in all samples and with all primer sets (Figure 3D). These results indicate that a probable fragmenta-

Figure 4. Southern blot analysis of 2-year-old HBV transgenic mice liver tumors. **T1** to **T7**, samples from seven different liver tumors from seven different mice; **L1** to **L3**, liver tissue surrounding the tumors; **+**, HBV transgenic mouse kidney positive control; and **-**, nontransgenic liver negative control. Approximately 20 μ g of extracted genomic DNA was digested with *Eco*RI, electrophoresed, transferred to Gene Screen, and probed with labeled PCR product (primer set II) to the HBV gene.

tion of the HBsAg gene has occurred in the foci but that the same transgene is intact in surrounding tissue that is able to produce HBsAg protein. Thus, altered DNA in the foci is the apparent explanation for the loss of HBsAg mRNA and protein expression in these cells.

Sequencing of Focal Amplicons

All amplicons produced using focal DNA were excised from gels and cycle-sequenced. In every case, an exact match with the HBsAg transgene was observed. These results suggest that alterations in the HBsAg transgene in foci, as evidenced by the absence of product, were due to larger-scale deletions as opposed to single-site mutations.

The HBV Gene in HCC from 2-Year-Old Mice Is Intact

Southern blot analysis was performed using DNA extracted from fresh liver tumors and kidney from 2-year-old transgenic HBsAg mice (Figure 4). A 5-kb DNA fragment was identified in the *Eco*RI digest using HBV Set II primer-derived PCR product as a probe. Samples from seven tumors (T1 to T7) obtained from seven separate mice and samples of liver surrounding the tumors (L1 to L3) and a kidney tissue positive control (+) showed the same-sized HBsAg DNA band. The liver sample from normal B6 mouse (-) was negative for the HBsAg gene. Thus, the *alb/psx* transgene in the HCC from livers of the 2-year-old mice appears to be intact.

Discussion

The present study suggests a separate cellular origin of regenerative nodules and HCCs in transgenic hepatitis B mice. There is loss of HBV-specific antigen in regenerating foci and nodules in the liver preceding the development of HBsAg-negative HCCs. This observation and other similarities between nodules and HCCs⁴³ support the conclusion that the HBsAg-negative HCCs arise from such negative foci.^{33–36,43} However, the present studies show that the regenerative nodules show deletions in the inserted *alb/psx* transgene, whereas HCCs contain the intact transgene, suggesting separate origins for most regenerating nodules and for HCCs.

In their initial studies, Chisari et al³³ observed that 50–4 transgenic HBsAg mice exhibited reduced expression of the HBsAg gene with increasing age and that most nodules in these mice lost expression of the HBV gene. Southern blot analysis revealed no difference between the genetic makeup of the DNA in the tumors and that of the tail DNA of the transgenic HBsAg mice³³ but a major decrease in the level of HBV mRNA.³⁴ Essentially all of the HCCs that were examined by histological staining did not contain HBsAg.^{34,35} Thus, despite the apparent integrity of the *alb/psx* transgene in HCCs, the HBsAg is not expressed in HCCs.

On the other hand, our PCR analysis of immunohistochemically negative areas of the nodules in liver tissue sections from 9-month-old transgenic HBV mice using primers directed against the promoter and pre-S1 region reveal that part of the *alb/psx* transgene is missing in eight of nine HBsAg-negative nodules. Thus, we conclude that deletion of DNA in the regenerating nodules is the apparent explanation of the loss of HBsAg mRNA and protein expression in most of the regenerative nodules in the transgenic *alb/psx* mice. It is not clear how this loss of HBV gene occurs or how it correlates with subsequent cancer formation. In most cases, these HBsAg-nonexpressing cells appear to function as normal hepatocytes, and they eventually may replace the cells damaged by storage of HBsAg.^{38,42} Overall, two types of DNA changes may occur, one giving rise to regenerating nodules (alteration of the transgene) and the other resulting in HCC with no alteration of the transgene. The facts that only one of nine nodules has an intact transgene and that HCCs have intact transgenes may be related to the finding that in this model of hepatocarcinogenesis, as well as in experimental hepatocarcinogenesis, there may be hundreds of nodules in one liver, but only one to three HCCs arise.⁴⁴ Thus, the nodules with the intact transgene may be the ones that will become HCCs.

The HCCs in these mice are slow growing and do not metastasize unless the mice are exposed to a carcinogen, such as aflatoxin.^{42,45} Co-carcinogens released from the area of cell death, such as reactive oxygen species,^{46,47} may also contribute to transformation and growth of the tumor cells, perhaps even contributing to loss of HBsAg expression despite the apparent integrity of the transgene.^{46–48} During this process, some progenitor cells in the liver most likely develop additional changes leading to HCC, particularly in male mice. The genetic nature of the putative changes in the HCC is not yet known, because neither alterations in the inserted *alb/psx* transgene nor mutations in specific oncogenes or suppressor genes have been identified in the HCCs arising in these mice.^{49,50}

The involvement of liver progenitor cells in the generation of nodules and HCC is supported by the microscopic location of newly developing lesions. Our present study shows proximity of HBsAg-negative foci and nodules to portal zones, where putative liver stem cells (oval cells) are located.^{51–53} In addition, microcarcinomas appear to develop adjacent to portal areas. For example, the arrows in Figure 1F point to the border between an HBsAg-negative microcarcinoma (top) and an HBsAg-

negative nodule. We performed three-dimensional analysis with a 20-section series to elucidate a possible role of cells within the biliary tree in the formation of the HBsAg-negative foci. We conclude that there is continued regeneration of antigen-negative foci from liver progenitor cells within or adjacent to the biliary system. These cells are stimulated to proliferate because of the loss of mature hepatocytes and decreased ability of mature hepatocytes to proliferate due the HBsAg storage injury. Survival of the newly formed hepatocytes is accomplished by alteration of the *alb/psx* transgene so that it is not expressed. During this process neoplastic nodules of various grades appear with development after 15 months of surface antigen-negative HCCs. Females living longer than 18 months appear able to regenerate large zones of apparently normal HBsAg-negative liver and to reconstitute their livers with normal appearing cells,³⁸ similar to what occurs in uPA transgenic mice.^{54,55} We postulate that liver regeneration is accomplished through a continued proliferation of "immature" hepatocytes or tissue-determined stem cells and that these gradually replace the injured surface antigen-expressing hepatocytes.

The observation that foci and nodules arise from the portal zone is consistent with the possibility that these newly generated tissues arise from bone marrow precursors.^{56–58} However, Vig and co-workers⁵⁹ did not find bone marrow donor-derived foci or nodules in female 6-month-old transgenic mice that had been irradiated and transplanted with male bone marrow. Similarly, we have not identified bone marrow-derived foci, nodules, or HCCs when the mice female transgenic HBsAg mice reconstituted with male bone marrow are sacrificed at 18 to 22 months of age (I. Guest, Z. Ilic, and S. Sell, unpublished data).

The expression of HBsAg in liver cells and HCC in humans with HBV infection has a completely different mechanism and meaning from that in the transgenic HBV mice. HBsAg was detected in about one-third of HCCs arising in humans who were serologically positive for HBV.⁶⁰ This contrasts with the lack of expression of HBsAg in essentially 100% of the HCCs in the transgenic *alb/psx* mice. In humans, HBsAg expression reflects infection of the cells with HBV^{60,61}; in the transgenic mice, expression is due to transcriptional activation of the *alb/psx* transgene.^{32–36} Thus, expression of the HBsAg reflects infection of the HCC cells with HBV. The mechanism of liver cell damage in humans with HBV infection is killing of infected hepatocytes by cytotoxic T lymphocytes reacting to viral antigens.³⁶ On the other hand, expression of HBsAg in the transgenic HBV mice is directly responsible for death of the transgenic hepatocytes, with little associated inflammation.

A comparison of the pathogenesis of HCC in humans with that in transgenic HBsAg mice also indicates major differences.^{36,61} The development of HCC in humans is attributed to several different mechanisms, including 1) deregulation of cell growth control genes by viral genes that have integrated into hepatocytes,^{18–24,62–65} 2) oxygen radical-mediated damage to DNA secondary to activated macrophages recruited in response to immune

inflammation,^{18,20,36,61} 3) regeneration resulting in proliferation of hepatic precursor cells,^{41,51,60} and 4) activity of the viral transactivating protein (HBx).^{18,23,27–31,66–68} Because there is no evidence for production of HBx or for integration of HBV DNA other than the *alb/psx* transgene in the transgenic HBV mice, the pathogenic pathways in common between the transgenic mice and human HBV-associated HCC are chronic liver injury, oxygen radical production, and proliferation of hepatocytes and liver progenitor cells.^{33,42,61}

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