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Hepatocarcinogenesis Due to Chronic Liver Cell Injury in Hepatitis B Virus Transgenic Mice[1]

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ABSTRACT

Fifty-nine transgenic mice from a lineage that overproduces the hepatitis B virus large envelope polypeptide and accumulates high intrahepatic concentrations of hepatitis B surface antigen were followed for evidence of liver disease throughout their 24-month life span. By 4 months of age all mice displayed biochemical and histological evidence of moderately severe chronic hepatitis which was followed sequentially by the development of regenerative nodules and oval cell hyperplasia (by 6 months), liver cell adenomas (by 8 months), and hepatocellular carcinomas (by 12 months of age). One hundred % of mice in this lineage developed hepatocellular carcinoma by 20 months of age, whereas no histopathological changes were observed in age- and sex-matched nontransgenic littermate controls over the same period of observation. These results indicate that overproduction of the hepatitis B virus large envelope polypeptide initiates a process characterized by liver cell injury, inflammation, and regenerative hyperplasia, which places large numbers of hepatocytes at risk for the development of transforming mutations, and inexorably progresses to hepatocellular carcinoma. We suggest that this is a general mechanism of hepatocarcinogenesis that may be operative in human hepatitis B virus infection and other necroinflammatory liver diseases as well.

INTRODUCTION

The strong epidemiological relationship (1, 2) between chronic hepatitis B virus infection and hepatocellular carcinoma corresponds to the presence of integrated HBV[3] DNA sequences in virtually all HBV-related HCC studied thus far (3–6). Although rearrangement of the integrated HBV DNA and flanking cellular sequences is common (reviewed in Ref. 7), and chromosomal deletions and translocations have been observed in human HCC (8, 9), insertional activation of cellular oncogenes is a rare event in this disease (10, 11). Similarly, insertional mutagenesis appears to be a relatively rare mechanism in HCC that occurs in woodchuck (12, 13), ground squirrel (14, 15), and Pekin duck (16, 17) hepatitis virus models. Thus, other mechanisms must be responsible for acquisition of the malignant phenotype in chronic HBV infection. In view of the prolonged interval between the onset of infection and the development of HCC it is very unlikely that HBV is a directly oncogenic tumor virus. Since HCC is preceded by chronic liver cell injury and inflammation in all of the hepadnavirus models (18), it is possible that the virus serves to initiate a complex series of events which cooperate to produce the malignant phenotype. We have previously reported the development of hepatocellular carcinoma (19) in transgenic mice that sustain prolonged liver cell injury and inflammation (20) due to overproduction of the HBV large envelope polypeptide (21) which causes the formation of long nonsecretable HBsAg filaments that accumulate within the endoplasmic reticulum of the hepatocyte and ultimately kill the cell. The histopathological features of the attendant liver disease in the most severely affected transgenic lineage are described in the current report. The development of hepatomas in virtually all of these trangenic mice provides compelling evidence that chronic liver cell injury is sufficient to cause HCC in this model, and it strongly suggests that HBV may be oncogenic in humans by virtue of its ability to induce chronic liver disease rather than by insertional mutagenesis. It also indicates that exogenous, chemical cofactors are not required for hepatocarcinogenesis in this disease.

MATERIALS AND METHODS

Production of Transgenic Mice. Transgenic mice lineage 50-4 [current designation, Tg(Alb-1 HBV)Bri 44] containing the HBV *Bgl*II-A fragment downstream of the albumin promoter was derived from parents, each of which were first generation (F_1) hybrids of the inbred mouse strains C57BL/6 and SJL as described previously (19–22). The lineage was expanded principally by backcrossing against the C57BL/6 parental strain and in some instances against nontransgenic littermates. All animals used in this study were hemizygous for the transgene.

Mice were maintained under code and were examined at quarterly intervals under metophane anesthesia for abdominal masses. Selected animals were euthanized at monthly intervals from 1 to 23 months. A total of 9 control nontransgenic mice were sacrificed at 3, 11, 18, and 24 months.

Histology. Tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. All sections were examined independently by H. A. D. and F. V. C. Histopathological diagnoses were based upon criteria described by Frith and Ward (23) and Ward (24).

Localization of HBsAg. HBsAg was localized in formalin-fixed paraffin-embedded tissue with Victoria blue stain as described previously (20).

AFP. Serum AFP was measured by radioimmunoassay as previously described (25). Tissue localization of AFP was performed on paraffin-embedded tissue sections by using rabbit anti-mouse AFP (ICN) at 1:100 dilution and the Vector Elite ABC kit.

RESULTS

Fig. 1 and Tables 1 and 2 summarize the incidence of tumors which appeared in the transgenic mice. True incidence is not represented here, inasmuch as many mice were sacrificed at earlier time points to document the chronic hepatitis. However, all mice older than 18 months had liver tumors. Males had more tumors than females, and at an earlier age. Fig. 2 demonstrates the gross comparison between normal nontransgenic littermates and transgenic tumor-bearing littermates. In spite of the large size of some of the liver tumors, some of which were hemorrhagic, were necrotic, and produced bloody ascites, no distant metastases were found.

Histology. The nontransgenic mice exhibited normal histology at all times examined (3 to 24 months). Liver sections from transgenic mice were normal at 1 month. After the second month there were areas of individual hepatocellular necrosis

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[3] The abbreviations used are: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; PHC, primary hepatocellular carcinoma.

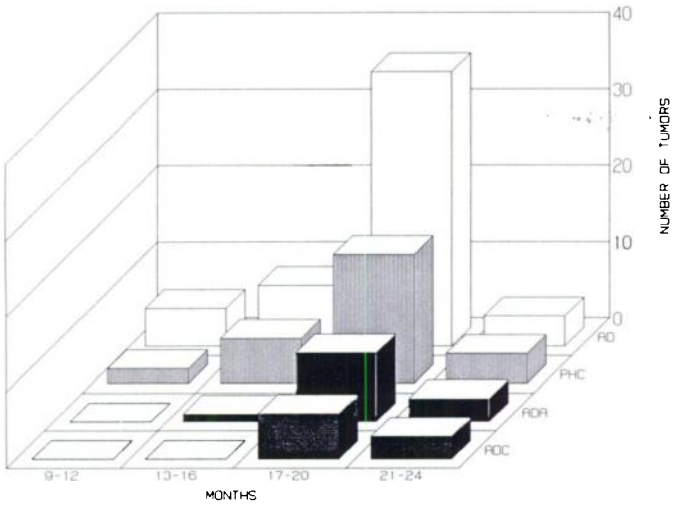

Fig. 1. Occurrence of adenomas and hepatomas in transgenic mice. *AD*, adenomas; *ADA*, adenomas with atypia; *ADC*, clear cell adenomas; *PHC*, hepatomas.

with inflammation. By the third month there was an accumulation of eosinophilic granular material in the cytoplasm of all hepatocytes (Fig. 3*B*) and more advanced changes of chronic hepatitis consisting of hepatocellular necrosis, Kupffer cell hyperplasia, and mononuclear cell infiltrate (Fig. 3, *C* and *D*). The chronic hepatitis induced by the envelope protein was only moderately severe histologically, perhaps similar to a persistent hepatitis in humans. Bridging necrosis was not seen, nor was there a marked portal inflammatory infiltrate.

This "injury" pattern persisted through the remaining months in the liver adjacent to preneoplastic lesions which appeared in the liver beginning with the seventh month. These lesions consisted at first of nodular areas of hepatocytes with clear cytoplasm (Fig. 4*B*) without compression of adjacent hepatocytes consistent with altered foci. Later nodular masses of hepatocytes appeared with compression of adjacent hepatocytes consistent with nodular hyperplasia, or regenerating nodules (Fig. 4*C*). Adjacent hepatocytes continued to demonstrate eosinophilic cytoplasm and nuclear inclusions, as well as areas of dysplastic hepatocytes (Fig. 4*C*). Other preneoplastic changes similar to those seen in chemical carcinogenic protocols were present, including irregular areas of smaller basophilic hepatocytes and bile duct proliferation at times resembling oval cell proliferation (Fig. 4, *A* and *D*).

Adenomas. Neoplastic masses of hepatocytes which compressed the adjacent parenchyma were scored as adenomas. Seventy-five adenomas were observed in 18 mice. These lesions appeared in mice beginning at 8 months and peaked in incidence around the 17th month (Fig. 1). Most adenomas consisted of solid masses of hepatocytes which were equal to or larger than normal hepatocytes and were arranged in distorted trabeculae which were only one to two cells thick (Fig. 5*A*). Several adenomas contained areas of abnormal trabecular differentiation (Fig. 5*B*). In others there were areas of nuclear pleomorphism with markedly enlarged nuclei (Fig. 5*C*) and increased numbers of mitotic figures. Although these areas may represent emerging microcarcinomas, there lesions were scored as adenomas with the abnormal histology involving less than 50% of the mass. Vascular invasion was not seen in any of the adenomas, although serial sections of each lesion was not performed. A smaller group of adenomas appeared in the older animals consisting of large hepatocytes with clear cytoplasm (clear cell adenomas) (Fig. 5*D*).

Hepatocellular Carcinomas (PHC). PHC were diagnosed when the neoplastic mass had a significant degree of abnormal trabecular differentiation (greater than 50%) consisting of trabeculae thicker than 2 cells (Fig. 6*A*). Solid carcinomas consisting of enlarged pleomorphic eosinophilic hepatocytes with markedly enlarged and dysplastic nuclei and both frequent and abnormal mitoses were also classified as PHC (Fig. 6*B*). Portal vein invasion, another evidence of aggressive behavior accepted as a criteria of malignancy, was documented in four PHCs (Fig. 6*C*). Eight PHCs were found to have arisen from within adenomas (Fig. 6*D*).

Victoria Blue Staining. Victoria blue positive HBV envelope protein was found in all hepatocytes by the second to third months (Fig. 7*A*). From the third to seventh month the Victoria blue positive HBV envelope protein continued to be expressed in most hepatocytes, but foci of Victoria blue negative staining cells were seen by the seventh month, probably corresponding the foci of altered cells and neoplastic nodules (Fig. 7*B*). All adenomas and hepatomas were Victoria blue negative (Fig. 7*C*) with the exception of one adenoma seen at 10 months which was filled with Victoria blue positive ground glass cells.

Serum AFP Levels. Table 1 summarizes the serum AFP levels for each group of tumors. There was a trend for serum AFP to be elevated in the older animals with tumor. Both animals with adenomas and hepatomas demonstrated elevated AFP.

Histological Localization of AFP. Increased numbers of AFP positive hepatocytes were found within the injury pattern of the chronic hepatitis consistent with the increased cell turnover (Fig. 8*A*). AFP positive cells were also present in adenomas (Fig. 8*B*) and PHC (Fig. 8*C*) from animals with tumors and

Table 1 *Summary of tumors and AFP levels in transgenic mice*

Diagnosis	Sex	No. of mice[a]	Age (mo)	No. of adenomas	No. of PHC	Serum (range) AFP (μg/ml)
Normal	Female	8	1–4	0	0	0.038 (0.02–0.09)
	Male	18	1–18	0	0	0.06 (0.02–0.08)
	Total	26	1–18	0	0	0.079 (0.02–0.09)
Nontransgenic	Female	4	2–24	0	0	0.063 (0.05–0.06)
	Male	5	2–24	0	0	0.07[a]
	Total	9	2–24	0	0	0.098 (0.05–0.07)
Adenomas	Female	11	8–23	22	0	0.68 (0.03–1.4)
	Male	9	9–20	21	0	2.08 (0.07–15)
	Total	18	8–23	43	0	2.42 (0.03–15)
PHC	Female	5	15–20	19	8	2.26 (1.0–5.1)
	Male	10	12–21	13	21	0.64 (0.03–1.9)
	Total	15	12–21	32	29	1.2 (0.03–5.1)
Grand total		68		75	29	

[a] Serum AFP measured in only one mouse in this group.

Table 2 *Adenomas and hepatomas by age and sex*

Diagnosis	Sex	Time (mo) 9–12	13–16	17–20	21–24	Totals
AD[a]	Female	1	3	24	3	31
	Male	4	5	12	1	22
	Total	5	8	36	4	53
ADA	Female	0	0	2	3	5
	Male	0	1	7	0	8
	Total	0	1	9	3	13
ADC	Female	0	0	3	3	6
	Male	0	0	3	0	3
	Total	0	0	6	3	9
PHC	Female	0	1	7	0	8
	Male	2	5	10	4	21
	Total	2	6	17	4	29

[a] AD, adenoma; ADA, adenoma with atypical histology; ADC, clear cell adenoma; PHC, hepatoma.

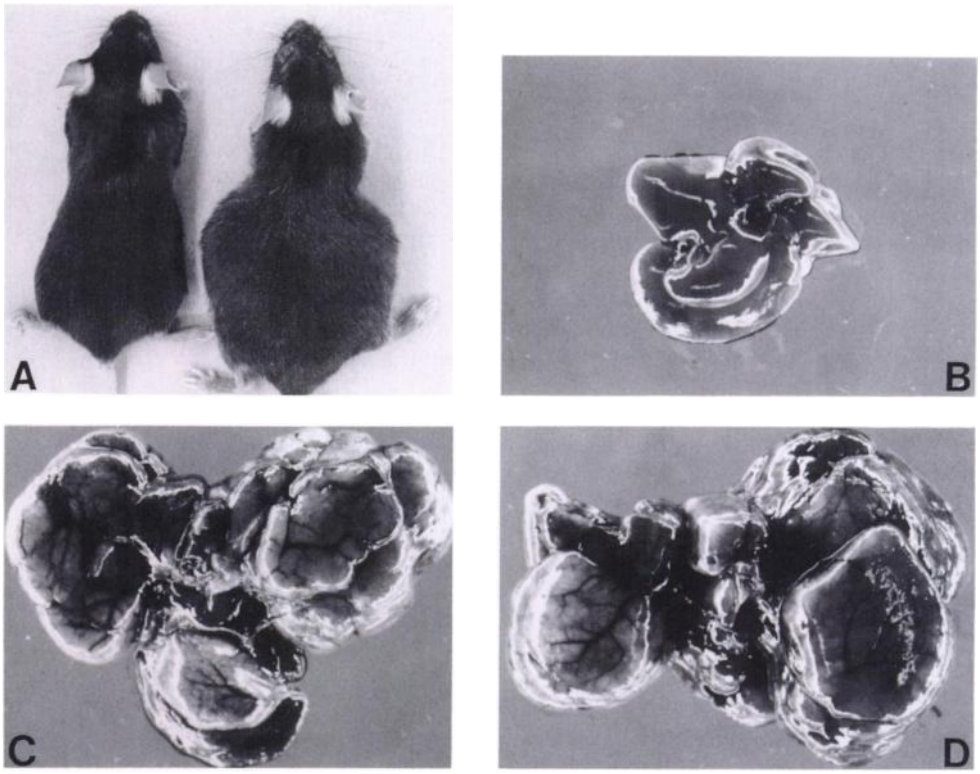

Fig. 2. Multiple, massive nodular hepatic neoplasms in transgenic mice. *A*, after 12 months of age trangenic mice developed palpable abdominal masses which progressively enlarged and caused massive abdominal distension (*A, right*) making them easily distinguishable from their nontransgenic littermates (*A, left*). *B*, normal liver from nontransgenic littermate. *C* and *D*, large palpable masses corresponded to the presence of multiple, large nodular, well vascularized hepatic tumors in transgenic mice.

high serum AFP levels. Only a few groups of cells were positive in the tumors, possibly suggesting that the production of AFP may be related to cell turnover.

DISCUSSION

These results suggest that overproduction of the HBV large envelope polypeptide initiates a series of events characterized by liver cell injury, regenerative hyperplasia and a secondary inflammatory response that inexorably progresses to the development of HCC in this transgenic mouse model. The progenitor mouse strains (C57BL/6 and SJL) used for these experiments do not spontaneously develop liver tumors. The complete lack of liver disease in the control nontransgenic mice during the same time period suggests that the C57BL/6 × SJL hybrids used for this study are also not high spontaneous tumor producers. The only difference between control and transgenic mice in this study was the presence of the HBV large envelope protein which accumulates at high, toxic concentrations in the endoplasmic reticulum and leads to the development of moderately severe chronic hepatitis with hepatocellular necrosis and increased cell turnover. Foci of vacuolated hepatocytes, regenerative or neoplastic nodules; basophilic foci; and oval cell proliferation were also present representing "preneoplastic changes" similar to the histological changes seen with chemical carcinogenic regimens in mice and rats (26, 27). The tumors produced were classified as adenomas or hepatomas by the current histological criteria. However, ploidy analysis demonstrated that most of these liver tumors, both adenomas and hepatomas, were aneuploid (19) suggesting that most of these liver tumors were actually malignant. The finding of microcarcinomas and carcinomas arising within adenomas is consistent with progression from the adenomas to hepatomas.

The nature of the early AFP containing lesions remains unclear, although we interpret them to be regenerative nodules. In the rat, during the early course of chemical hepatocarcinogenesis, a variety of AFP containing lesions appear (27). These include oval cells, tubular-like structures, and zones of atypical hyperplasia (28) each of which is a proliferative lesion, but different from regenerative nodules and possibly premalignant.

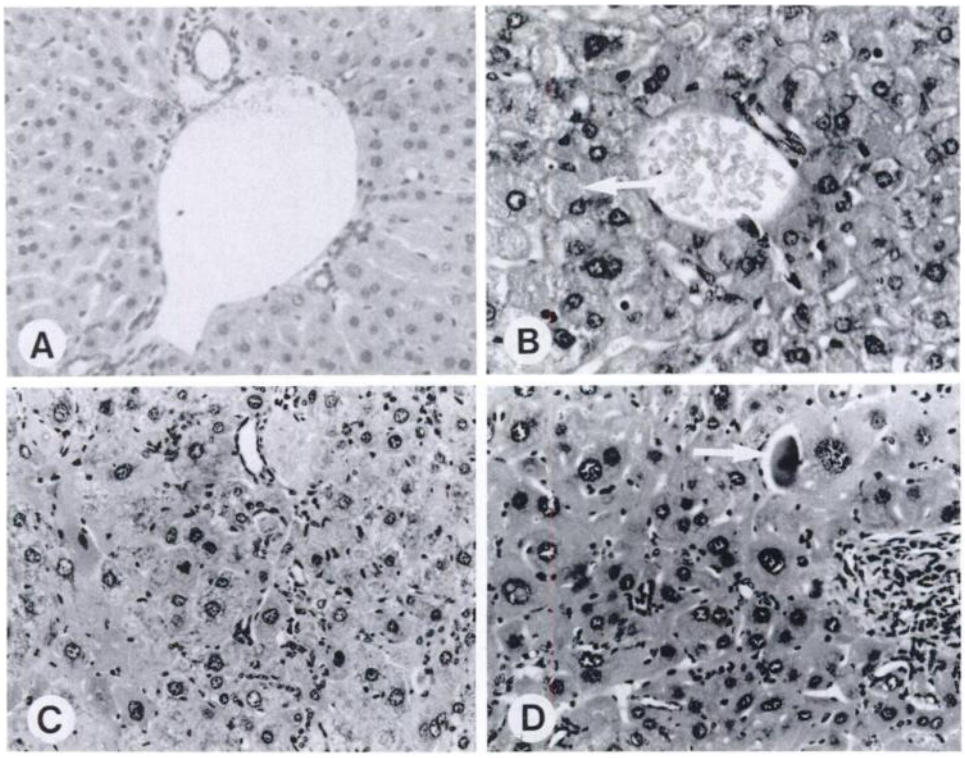

Fig. 3. Histological effect of HBV envelope protein in transgenic mice. *A*, normal liver in a non-transgenic control mouse at 18 months, H & E, × 200. *B*, ground glass cells in a transgenic mouse at 2.5 months; eosinophilic envelope material fills the cytoplasm of all the hepatocytes (*arrow*). H & E, × 400. *C*, chronic hepatitis in a trangenic mouse at 4 months. H & E, × 200. *D*, chronic hepatitis with persistent round cell infiltrate in the portal area (*right*) and dying liver cell (*arrow*). H & E, × 400.

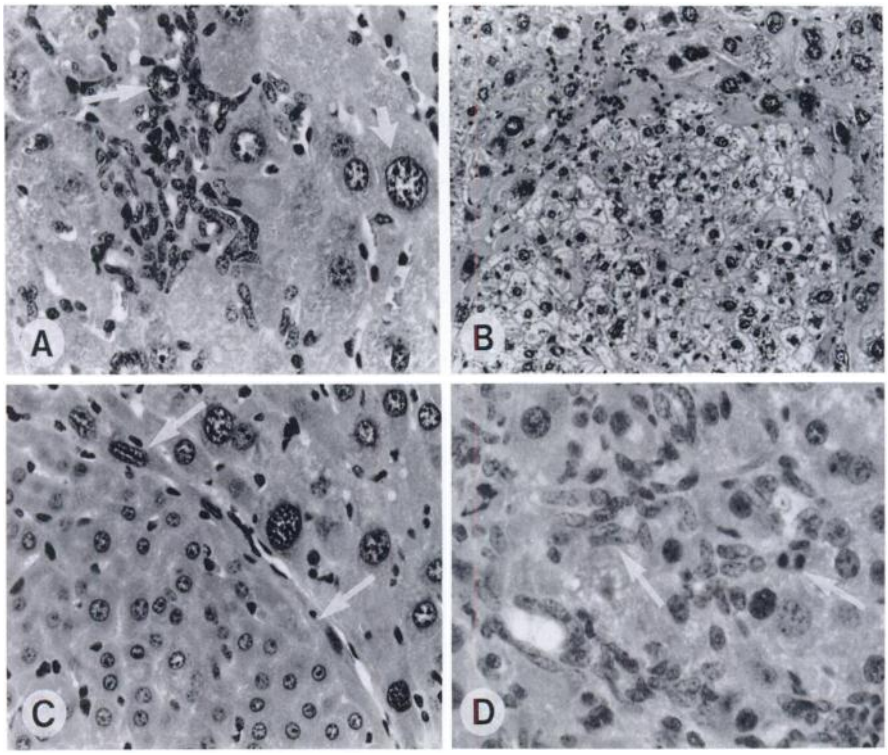

Fig. 4. Preneoplastic changes in transgenic mice. *A*, bile duct proliferation (*long arrow*) and dysplastic hepatocytes (*short arrow*) in a transgenic mouse at 8 months. H & E, × 400. *B*, foci of vacuolated cells in a transgenic mouse at 10 months. H & E, × 200. Note the ground glass appearance of the hepatocytes around the foci. *C*, neoplastic nodule in 24 month transgenic mouse. Note the bland uniformity of the hepatocytes in the nodule compared to the "normal" more dysplastic hepatocytes in the adjacent liver. Beginning compression of adjacent hepatocytes (*arrows*) suggests that this is a precursor of the adenomas which markedly compress the adjacent hepatocytes. H & E, × 400. *D*, marked proliferation of oval cells (*arrow*) which infiltrate between hepatocytes in a trangenic mouse at 20 months. H & E, × 400.

AFP positive regenerative nodules have not been identified in the rat. However, single AFP producing hepatocytes have been seen in both mouse (29, 30) and rat (31). The threshold of AFP production by adult hepatocytes is considerably lower in mice than in rats. The serum AFP level is much higher in mice (32, 33) than in rats (34) after liver injury or partial hepatectomy and cultures of adult mouse hepatocytes produce AFP more readily than those of rat hepatocytes during proliferation *in vitro*.[4] Thus the morphological appearance and the presence of AFP in some of the early lesions in the transgenic mice with liver injury are consistent with regenerative hyperplasia.

The data presented herein and earlier (19) are most compatible with the hypothesis that HBV is a complete carcinogen that causes HCC by initiating a complex series of events in response to chronic liver cell injury. Hepatocellular injury in this model is due to overexpression of the HBV large envelope polypeptide since neither liver cell injury nor HCC has been observed in other transgenic lineages that do not overexpress this viral peptide either at all or to the same degree (22, 35–

[4] H. L. Leffert, personal communication.

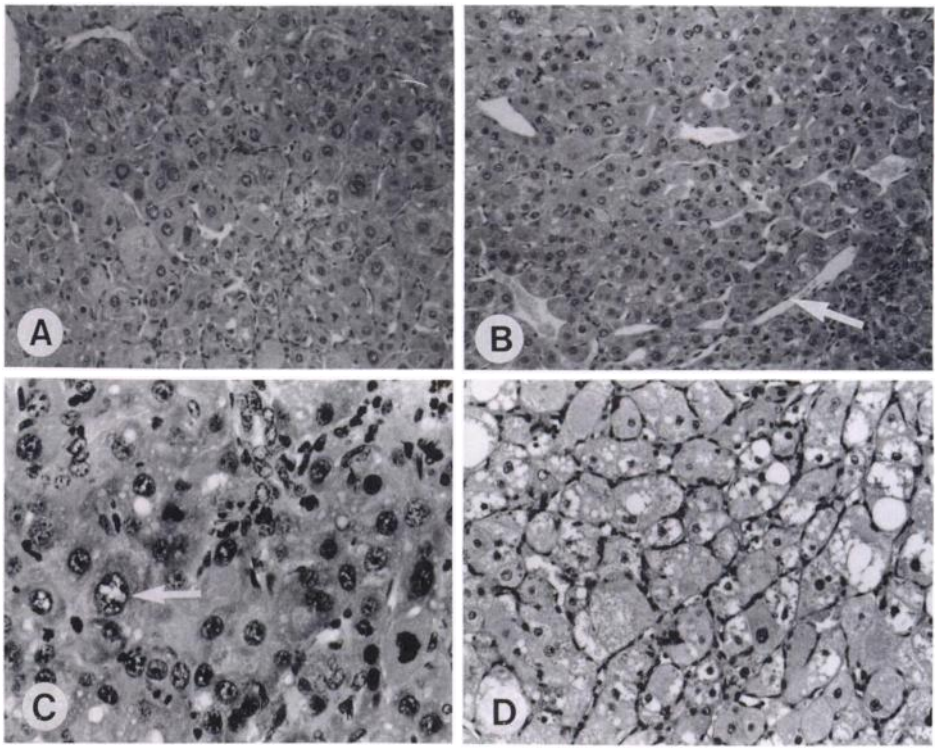

Fig. 5. Adenomas in transgenic mice. *A*, adenoma in a 22-month-old transgenic mouse. The trabeculae are identifiable but always one to two cells thick. H & E, × 200. *B*, adenoma in a 24-month mouse with areas of thickened trabeculae, possibly representing a microcarcinoma. H & E, × 200. *C*, a small area of marked cytological atypia (*arrow*) in an adenoma, also suggesting a microcarcinoma arising in the adenoma. H & E, × 400. *D*, clear cell adenoma. Each hepatocyte has a bland small nucleus and a foamy cytoplasm with vacuoles, suggesting a degenerative change. H & E, × 400.

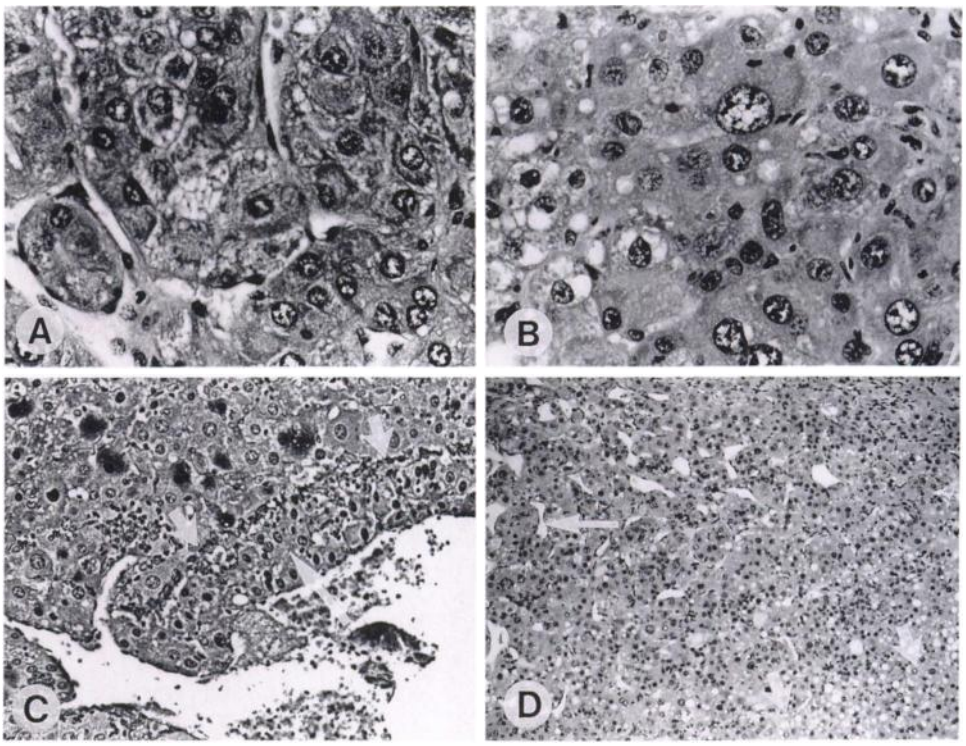

Fig. 6. Hepatomas in transgenic mice. *A*, PHC with large trabeculae three or more hepatocytes wide. H & E, × 400. *B*, solid PHC made up of cytologically malignant cells with eosinophilic cytoplasm. H & E, × 400. *C*, PHC with portal vein invasion. Note elastica of portal vein (*arrows*) with tumor growing in vessel lumen. Reticulum stain, × 200. *D*, PHC with abnormal trabeculae arising in adenoma (*arrow*) made up of smaller cells with normal nuclei and fat. H & E, × 100.

42). One might expect that hepatocytes that express low levels of the large envelope polypeptide would have a selective survival advantage in this model. If they were preferentially driven to regenerate in response to the loss of hepatocyte mass, they would run a correspondingly higher risk of acquisition of transforming mutations. Our observation that the preneoplastic modules and the tumors themselves display a marked reduction in expression of the large envelope polypeptide (19) is compatible with this hypothesis; however, it is also possible that the observed decrease in viral envelope polypeptide expression reflects changes in hepatocellular differentiation that are secondary to the transformation process.

In view of the association between chronic liver cell injury and HCC in a variety of other liver diseases (43–46), we believe

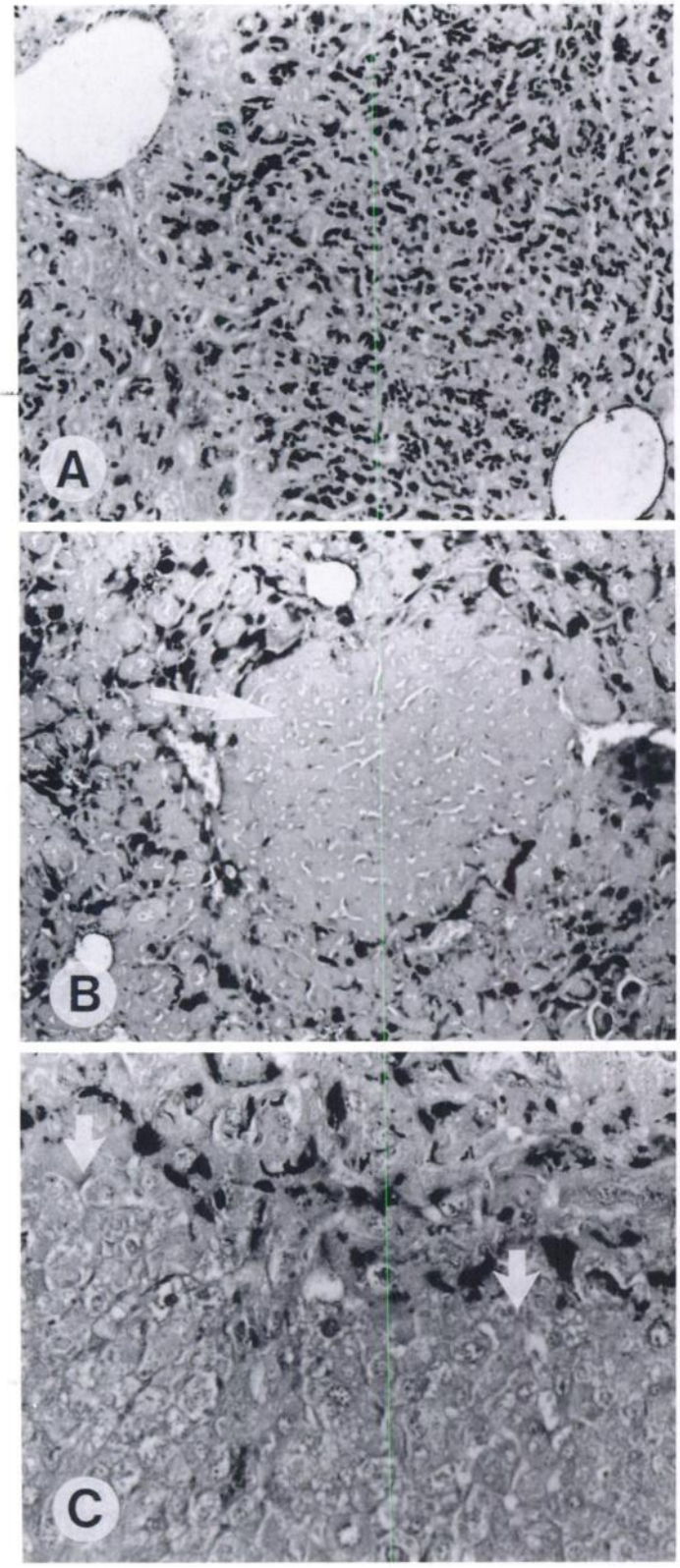

Fig. 7. Victoria blue staining of livers of transgenic mice. *A*, trangenic mouse 2.5 months; note all hepatocytes are positive. × 100. *B*, trangenic mouse at 7 months with area of negative hepatocytes (*arrow*), probably a precursor of adenomas. × 100. *C*, adenoma with negative staining (*arrows*) with positive staining of adjacent hepatocytes. × 200.

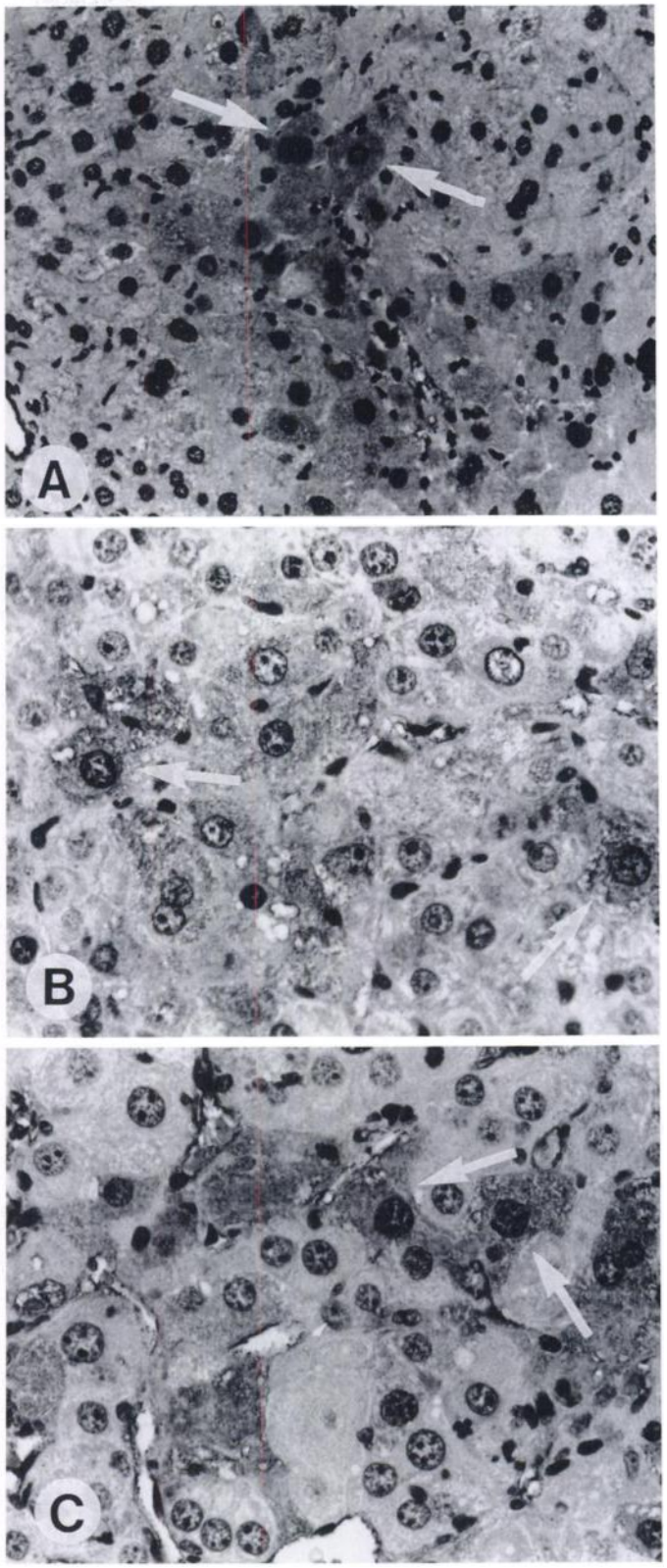

Fig. 8. AFP staining of livers of transgenic mice. *A*, transgenic mouse at 4 months with a group of AFP positive hepatocytes (*arrows*). × 200. *B*, transgenic mouse at 22 months with a few AFP positive adenoma cells (*arrows*). × 400. *C*, transgenic mouse at 22 months with AFP positive hepatoma cells (*arrows*). × 400.

that, regardless of etiology or pathogenesis, injury induced regeneration of large numbers of hepatocytes over a prolonged period of time creates a high risk for the development of multiple random mutations throughout the hepatocellular genome that eventually transform the cell. It is likely that hepatocellular regeneration alone may not be sufficient to induce these putative genetic lesions since HCC does not develop following partial hepatectomy nor does it develop in the rapidly

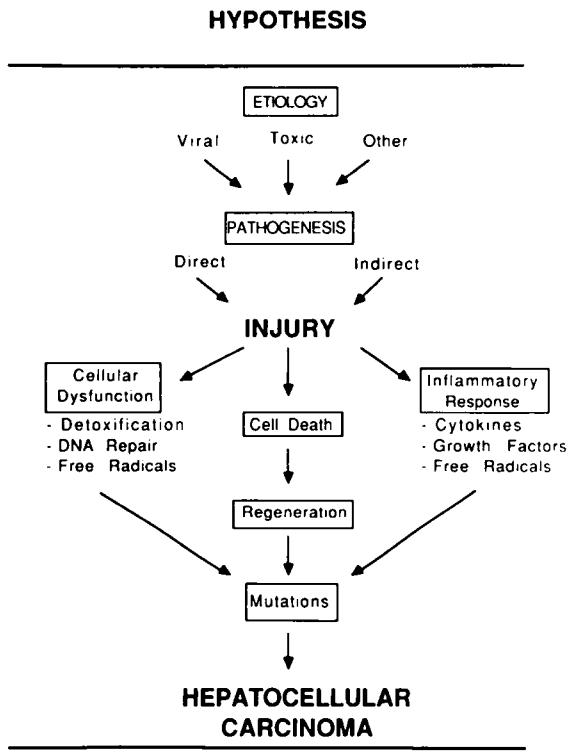

Fig. 9. Hypothesis. Irrespective of etiology or pathogenesis, chronic liver cell injury induces several events which cooperate to greatly increase the risk of transforming mutations leading to hepatocellular carcinoma. In this model the metabolic consequences of cellular dysfunction and inflammation serve as cofactors to increase the mutation rate in regenerating hepatocytes.

proliferating fetal liver. Thus, additional cofactors are probably needed for the development of HCC in this setting. Such cofactors might include mutagenic products of the associated inflammatory cells or impaired detoxification or DNA repair mechanisms within the injured hepatocyte, as illustrated in Fig. 9.

In summary, we have demonstrated that overproduction of the HBV large envelope polypeptide in transgenic mice causes the formation of nonsecretable HBsAg filaments which accumulate within the endoplasmic reticulum of the hepatocyte and eventually kill the cell. We have also shown that the attendant chronic hepatitis inevitably progresses to hepatocellular carcinoma. We believe the transforming cellular events that are set in motion as a consequence of liver cell injury in this model are also likely to occur in other necroinflammatory diseases that precede HCC. Identification of the cellular cofactors involved in hepatocarcinogenesis and establishment of therapeutic strategies to interrupt these events may derive from further analysis of the molecular pathogenesis of HCC in this transgenic mouse model.

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