

Hepatitis B Virus Replication Is Cell Cycle Independent during Liver Regeneration in Transgenic Mice†

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Received 19 December 1996/Accepted 27 February 1997

The content of hepatitis B virus (HBV) replicative forms and HBV core protein in the liver of HBV transgenic mice is transiently reduced during massive liver regeneration following partial hepatectomy while the steady-state content of viral RNA is unchanged. This antiviral effect is triggered by interferon and tumor necrosis factor that are induced in the liver following hepatectomy and either prevent the formation or accelerate the degradation of viral nucleocapsids in the cytoplasm of the hepatocyte. Despite massive hepatocellular turnover, this effect is independent of liver cell division, indicating that HBV replicates efficiently in resting and dividing hepatocytes.

Hepatitis B virus (HBV) is a noncytopathic virus that causes a necroinflammatory liver disease characterized by a lymphomononuclear inflammatory cell infiltrate and Kupffer cell hyperplasia (22). Viral clearance and liver disease during acute HBV infection are associated with a strong, polyclonal and multispecific cytotoxic T-cell (CTL) response, which is weak or not detectable in patients with chronic hepatitis who do not clear the virus (2). HBV-specific CTL have been shown to cause a necroinflammatory liver disease when they are injected into HBV transgenic mice (6, 9), and the same CTL have been shown to clear HBV RNAs, proteins, nucleocapsid particles, and DNA replicative intermediates from the liver and virions from the serum of these animals, mostly by noncytopathic mechanisms (9). The antiviral properties of the CTL are mediated by the intrahepatic induction of inflammatory cytokines such as gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α) that are released in the liver by the antigen-activated CTL (6, 9).

These observations suggest that, in addition to destroying HBV-infected hepatocytes during viral hepatitis, a strong intrahepatic immune response to HBV may suppress viral replication and possibly even clear the virus from the liver noncytopathically, in effect curing the infection. In support of this hypothesis, it has been reported that the woodchuck hepatitis virus is cleared from the acutely infected woodchuck liver without massive hepatic necrosis or liver cell regeneration (15), suggesting that noncytolytic clearance mechanisms may also occur during woodchuck hepatitis virus infection. Recent observations indicate that at least two intracellular pathways might be activated by CTL and inflammatory cytokines, one that posttranscriptionally degrades the viral RNA (12, 21), and another that eliminates the nucleocapsid particles and their content of HBV replicative intermediates in the cytoplasm of "cytokine-activated" hepatocytes (9).

It has been previously reported that clearance of the duck hepatitis B virus (DHBV) is associated with hepatocellular regeneration in models of acute and chronic DHBV infection

(4, 14). The investigators ascribed the clearance of virus to the process of hepatocellular regeneration. This is compatible with observations suggesting that HBV replication is cell cycle dependent in transfected human hepatoma cell lines (19). However, the presence of intrahepatic inflammatory cells and Kupffer cell hyperplasia in the DHBV model could also have contributed to the reported antiviral effect. The availability of transgenic mice that replicate HBV at high levels in the liver (11) created the opportunity to directly examine the extent to which hepatocellular regeneration affects HBV replication in the absence of an inflammatory response.

Kinetics of hepatocyte turnover. To monitor the effect of hepatocyte turnover on HBV replication in this model, 12- to 9-week-old male mice from lineage 1.3.46 (official designation, Tg[HBV 1.3 genome]Chi46) (11) and lineage 1.3.32 (official designation, Tg[HBV 1.3 genome]Chi32) (11) were subjected to 70% partial hepatectomy (PH) as previously described (10) and sacrificed at multiple time points after surgery. Because similar results were obtained in both lineages, representative results from lineage 1.3.46 will be described in this report. To determine the extent of hepatocyte turnover after 70% PH, the distribution of proliferating cell nuclear antigen (PCNA), a marker of cells in S phase (16), was assessed by immunohistochemical analysis exactly as described previously (10). As shown in Fig. 1A (inset), virtually all hepatocytes in the resting liver are negative by this technique. However, more than 90% of the hepatocyte nuclei were PCNA positive when they were examined 36 h (data not shown), 60 h (Fig. 1B, inset), and 72 h (Fig. 1D, inset) after 70% PH. Five days after 70% PH, the number of PCNA-positive cells was greatly diminished (data not shown), indicating that the vast majority of the hepatocytes progressed beyond S phase and probably beyond mitosis. The distribution of PCNA returned to normal levels (less than 0.1%) 14 days after PH (data not shown). These observations were confirmed by quantitation of the hepatocellular nuclear labeling index (LI) in liver sections of mice that received a single intraperitoneal injection (2.5 μ g/mouse) of bromodeoxyuridine (BrdU; Sigma, St. Louis, Mo.) 36 h after PH (Table 1). The hepatocyte LI was expressed as the percentage of total hepatocytes containing BrdU-positive nuclei as previously described (13).

Serum alanine transaminase (sALT) activity was measured as previously described (9), and liver histopathology was mon-

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† Manuscript no. 9642-MEM from the Scripps Research Institute.

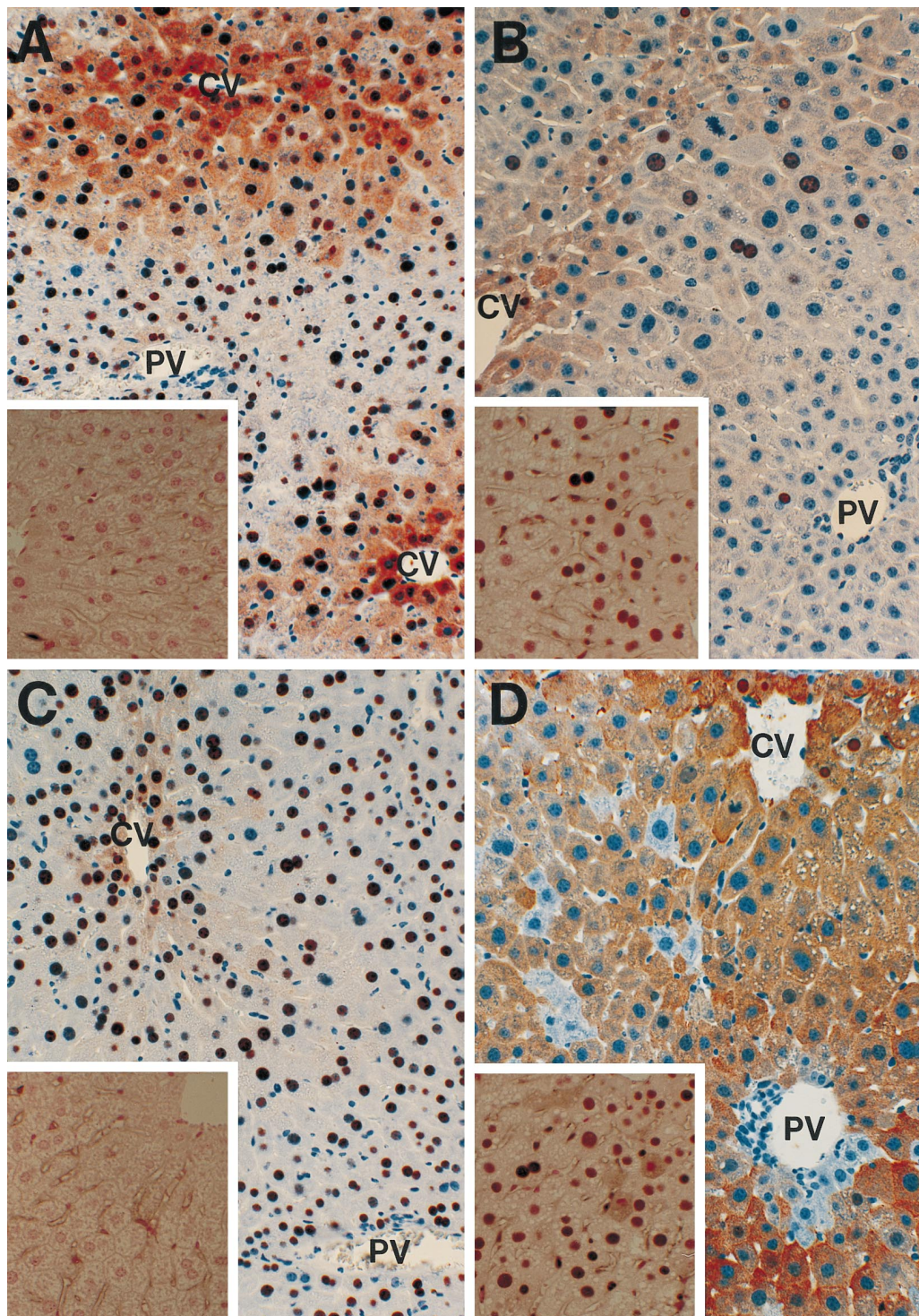


FIG. 1. Intracellular localization of HBV core protein and PCNA following 70% PH. Liver sections obtained from age- and hepatitis B surface antigen (HBsAg)-matched transgenic males (lineage 1.3.46) were stained for the presence of PCNA (insets) and HBc/eAg. Stained sections from resting liver (A), livers harvested 60 h after 70% PH (B and C), and a liver harvested 72 h after 70% PH (D) are shown. Animals for panel B received five saline injections (200 μ l each), one every 12 h starting at the time of surgery. Animals for panel C received five injections of recombinant human TGF- β (10 μ g each), one every 12 h starting at the time of surgery. Animals for panel D received intraperitoneally a cocktail of anticytokine antibodies 6 h before surgery. Hamster monoclonal antibodies specific for mouse IFN- γ (250 μ g/mouse), TNF- α (250 μ g/mouse), and neutralizing sheep Ig to mouse IFN- α/β (300 μ l/mouse) were used in this study. Central (CV) and portal (PV) veins are indicated. (Immunoperoxidase stain for PCNA and HBc/eAg; magnification \times 200).

TABLE 1. Analysis of serum alanine transaminase activity and hepatic BrdU-positive nuclei following 70% PH

| Time after 70% PH | sALT activity (U/liter) ^a | Hepatocyte LI (%) ^b |
|-------------------|--------------------------------------|--------------------------------|
| 0 h..... | 24 ± 14 | 0.07 ± 0.02 |
| 36 h..... | 467 ± 95 | ND ^c |
| 48 h..... | 328 ± 77 | 64 ± 12 |
| 60 h..... | 284 ± 42 | 60 ± 7, 2 ± 7 ^d |
| 72 h..... | 58 ± 23 | 56 ± 14, 61 ± 6 ^e |
| Day 5..... | 46 ± 12 | ND |
| Day 7..... | 32 ± 8 | ND |
| Day 10..... | 42 ± 15 | ND |

^a Values are mean activities ± standard errors of the mean.

^b Data are mean percentages of total hepatocytes containing BrdU-positive nuclei ± standard errors of the mean (13).

^c ND, not done.

^d Mean percentage of BrdU-positive nuclei for mice given TGF-β after PH.

^e Mean percentage of BrdU-positive nuclei for mice given a cocktail of antibodies to TNF-α, IFN-α/β, and IFN-γ before PH.

itored by hematoxylin and eosin staining of zinc-buffered formalin-fixed tissue sections as previously described (9). Serum alanine transaminase levels were modestly elevated at the time of autopsy only in mice that were sacrificed at 36, 48, and 60 h after PH (Table 1). By comparing these sALT profiles with known standard profiles (9) and with previously reported mouse models of liver disease (1, 9), it was determined that no more than 5% of the residual hepatocytes were injured or destroyed after 70% PH. Furthermore, the transient increase in sALT activity probably reflects enzyme release from necrotic liver tissue that could not be completely excised at the ligation site, rather than necrosis in the residual liver, because we did not detect any histological evidence of liver cell necrosis or inflammation at any time point in the regenerating livers (see below).

Effect of hepatocyte turnover on HBV RNA expression. To examine the effect of hepatocellular turnover on HBV RNA, total hepatic RNA was extracted (11) and hepatic HBV expression and glyceraldehyde-3-phosphate dehydrogenase expression in tissues obtained from the same animal at the time of surgery and at the time of autopsy were compared by Northern blot analysis as previously described (8). As shown in the Northern blot at the top of Fig. 2 for a representative mouse from each group, we observed little or no change in the hepatic steady-state content of the 3.5- and 2.1-kb HBV RNA at any time following PH compared to pre-PH levels (time 0) in the same animal.

Effect of hepatocyte turnover on HBV replication. To examine the effect of hepatocellular turnover on HBV replication, total hepatic DNA was extracted from the same livers and analyzed by Southern blot analysis exactly as previously described (11). As shown in the Southern blot in Fig. 2, the hepatic content of HBV DNA replicative forms decreased moderately between 3 and 7 days after PH, most noticeably on day 5. This reduction in HBV replicative forms was independent of their template, since the steady-state content of the pregenomic RNA was essentially normal at all time points (Fig. 2, top). By day 10, HBV replicative forms had almost returned to baseline compared to pre-PH levels (Fig. 2). This suggests that PH transiently inhibits HBV replication by activating a posttranscriptional antiviral process.

Effect of hepatocyte turnover on cytoplasmic HBcAg content. To determine the effect of hepatocyte turnover on hepatic hepatitis B core antigen (HBcAg) content, the distribution of HBcAg before and after PH was assessed by immunohistochemical analysis as previously described (10). As shown in

Fig. 1A, cytoplasmic HBcAg is detectable in the resting liver primarily in centrilobular hepatocytes. We have previously shown that the preferential expression of cytoplasmic HBcAg in centrilobular hepatocytes is associated with higher-level expression of the 3.5-kb HBV RNA in these cells than in periportal and lobular hepatocytes (11). Sixty hours after 70% PH, cytoplasmic HBcAg was detectable in only about 10% of the centrilobular hepatocytes (Fig. 1B), in parallel with the reduction of HBV DNA replicative forms (Fig. 3A, lanes 1 to 4). Cytoplasmic HBcAg was almost completely absent from the regenerating liver 72 h and 5 to 7 days after 70% PH (data not shown), concomitant with the more profound reduction of HBV DNA replicative forms (Fig. 2). The distribution of cytoplasmic HBcAg returned to normal between 10 and 14 days after PH (data not shown). These results indicate that cytoplasmic HBcAg and HBV replication were simultaneously reduced in centrilobular hepatocytes between 60 h and 7 days after 70% PH, and they returned to baseline simultaneously thereafter.

Effect of hepatocyte turnover on nuclear HBcAg content. As shown in Fig. 1A, more than 90% of hepatocyte nuclei are HBcAg positive. As previously reported, these cells contain HBV RNA and empty nucleocapsid particles in the nucleus (11). Furthermore, upon dissolution of the nuclear membrane during mitosis, empty nucleocapsid particles are released into the cytoplasm from which they do not reenter the nucleus (10). Concomitant with massive liver cell turnover, nuclear HBcAg almost completely vanished from the hepatocyte nuclei 60 h (Fig. 2B), 72 h, 5 days, and 7 days (data not shown) after 70% PH and returned to normal thereafter. In contrast, during this

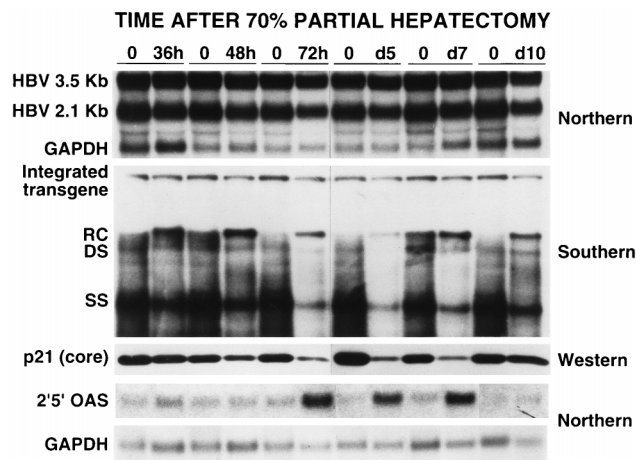


FIG. 2. Effect of 70% PH on HBV gene expression and replication in lineage 1.3.46. Twelve age- and HBsAg-matched transgenic males were subjected to 70% PH and sacrificed at multiple time points thereafter. Total liver RNA specimens (20 μg) isolated from biopsies taken from six representative mice were compared for the same animal at the time of surgery (time 0) and at the time of autopsy (as indicated) for the expression of HBV and 2'5' OAS mRNA by Northern blot analysis (as indicated). The housekeeping gene for glyceraldehyde-3-phosphate dehydrogenase was used to normalize the amount of mRNA bound to the membrane. Total liver DNA (20 μg) was isolated from the same livers and analyzed by Southern blotting (as indicated). All DNA samples were RNase treated before being analyzed by gel electrophoresis. Bands corresponding to the expected size of the integrated transgene, relaxed circular (RC), double-stranded linear (DS), and single-stranded (SS) HBV DNA are indicated. Bands corresponding to the integrated transgene can be used to normalize the amount of DNA bound to the membrane. The filters were hybridized with a ³²P-labeled HBV-specific DNA probe. Total liver protein (50 μg) was isolated from the same livers and analyzed by Western blotting for the expression of HBV core protein (p21), as indicated.

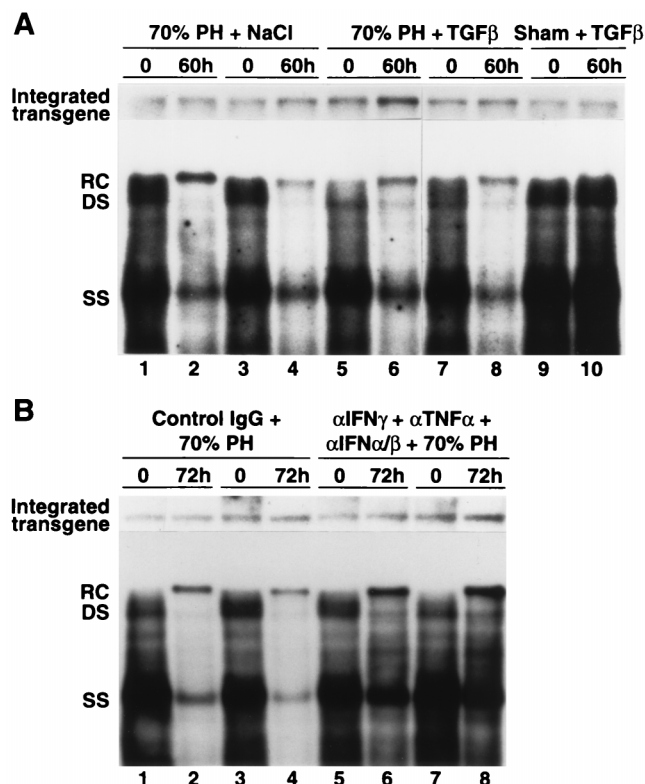


FIG. 3. The PH-dependent inhibition of HBV replication is independent of hepatocyte turnover and dependent on inflammatory cytokines. (A) Twelve age- and HBsAg-matched transgenic males from lineage 1.3.46 were subjected to 70% PH, injected with multiple doses of either saline or TGF- β , and sacrificed 60 h after surgery, as described in the legend to Fig. 1. In addition, three animals were injected with the same TGF- β treatment following sham operations and sacrificed 60 h after surgery. Southern blot analysis of 20 μ g of total liver DNA isolated from liver biopsies taken from two representative mice per group is shown. (B) Twelve age- and HBsAg-matched transgenic males from lineage 1.3.46 were subjected to 70% PH, injected with either a mixture of irrelevant control antibody (500 μ g of purified hamster IgG plus 300 μ l of normal sheep Ig per mouse) or a cocktail of anticytokine antibody and sacrificed 72 h after surgery, as described in the legend to Fig. 1. Southern blot analysis of 20 μ g of total liver DNA isolated from liver biopsies taken from two representative mice per group is shown. All DNA samples were RNase treated before being analyzed by gel electrophoresis. The filters were hybridized with a 32 P-labeled HBV-specific DNA probe. DNA loading was normalized by comparing the signal intensities of the integrated transgene DNA seen at the top of each lane.

entire period, hepatic HBV RNA content was unchanged (Fig. 2, top).

Since the absolute hepatic content of hepatitis B core/e antigen (HBc/eAg) was reduced (Fig. 2, Western blot), and since serum HBc/eAg concentration did not change (data not shown), the loss of hepatic HBcAg in the regenerating liver (Fig. 1B) is not due to its dilution in daughter cells nor to its secretion into the serum. Since HBV core protein accumulates in the resting liver at a given net rate (synthesis minus degradation), it would appear that this rate is perturbed in the regenerating liver either by enhanced degradation or by reduced synthesis.

Inhibition of HBV replication following 70% PH is independent of hepatocellular turnover. To determine whether the modest PH-dependent inhibition of HBV replication requires liver cell division, transgenic mice from lineage 1.3.46 were subjected to 70% PH and treated with multiple high doses of recombinant human transforming growth factor β (TGF- β) (kindly provided by Susan Kramer, Genentech Inc., San Fran-

cisco, Calif.), a known suppressor of liver cell regeneration (17). The animals were sacrificed 60 h after surgery, and the hepatic contents of HBcAg and HBV replicative forms were compared with those of control animals which received either multiple doses of saline after 70% PH or the same TGF- β treatment following sham operations. As shown in Fig. 1C and Table 1, TGF- β strongly suppressed liver cell division as demonstrated by the fact that virtually all hepatocytes were positive for nuclear HBcAg and negative for PCNA (inset) and BrdU (Table 1). In contrast, most hepatocyte nuclei in control mice that received saline were HBcAg negative (Fig. 1B), PCNA positive (Fig. 1B, inset), and BrdU positive (Table 1). Importantly, HBcAg was profoundly reduced in the cytoplasm of centrilobular hepatocytes of mice that received TGF- β after 70% PH (Fig. 1C) in contrast to resting centrilobular hepatocytes (Fig. 1A), indicating that this effect, triggered by PH, was independent of liver cell division.

To monitor whether HBV replication was similarly affected, the hepatic content of HBV replicative forms was analyzed by Southern blotting of samples from the same animals. As shown in Fig. 3A for two representative animals per group, a comparable reduction in HBV replicative forms was observed in mice that received either TGF- β (lanes 5 to 8) or saline (lanes 1 to 4) after 70% PH. In addition, no reduction in HBV replication was observed in animals that received the same TGF- β treatment after sham operations (lanes 9 and 10), demonstrating that TGF- β alone does not affect HBV replication.

Collectively, these results suggest that although PH activates an antiviral state in the hepatocyte that eliminates cytoplasmic core particles and their cargo of HBV replicative intermediates, this process is independent of hepatocyte turnover.

Inhibition of HBV replication following 70% PH is dependent on inflammatory cytokines. To determine whether the PH-dependent inhibition of HBV replication is dependent on inflammatory cytokines previously shown to downregulate HBV gene expression and replication (7, 9), we measured the intrahepatic content of a large panel of cytokines in the regenerating livers. Total RNA (10 μ g) was analyzed for cytokine expression by RNase protection using a multiprobe assay as previously described (9). With the exception of a moderate activation (about fivefold at 36 and 48 h and two- to threefold at the other time points) of TNF- α and interleukin 1 α (IL-1 α) expression (data not shown), we did not observe induction of any of the lymphokines (IL-2, IL-3, IL-4, IL-5, IFN- γ) or monokines (IL-1 β , IL-6) tested (data not shown). Total RNA (20 μ g) was also analyzed for the hepatic expression of 2'5'-oligoadenylate synthetase (2'5'-OAS) transcripts by Northern blotting as previously described (7). As shown in the Northern blot at the bottom of Fig. 2, this marker of IFN- α/β induction was moderately induced (three- to fivefold) at 72 h and at 5 to 7 days after 70% PH, concomitant with the reduction of HBV replicative intermediates and HBcAg, also shown in Fig. 2. The cellular origin of TNF- α , IL-1 α , and IFN- α/β in the regenerating liver is currently unknown, but it is likely that they are produced by intrahepatic macrophages that are also regenerating in these livers and may, therefore, be activated.

Next, we subjected transgenic mice from lineage 1.3.46 to 70% PH in the presence of a cocktail of neutralizing antibodies to TNF- α (20), IFN- α/β (5), and IFN- γ (18), cytokines that are known to inhibit HBV replication noncytopathically in this model (7, 9). The animals were sacrificed 72 h after PH, and the hepatic contents of HBcAg and HBV replicative forms were compared with those of control animals that received a mixture of purified hamster immunoglobulin G (IgG) (Jackson ImmunoResearch) and normal sheep Ig as control antibodies (7). As shown in Fig. 1D, a high level of hepatocyte turnover

was detected in the animals that received the anticytokine antibodies as demonstrated by the high percentage (over 90%) of hepatocytes containing HBcAg-negative nuclei that were PCNA positive (Fig. 1D, inset) and BrdU positive (Table 1). Importantly, cytoplasmic HBcAg was still present in the hepatocytes of these animals (Fig. 1D) while it was virtually absent from the animals that received irrelevant antibody (data not shown). The cytoplasmic staining in periportal hepatocytes is likely to reflect release of empty nuclear core particles into the cytoplasm during mitosis, while the cytoplasmic staining in centrilobular hepatocytes may also reflect the presence of mature core particles.

To monitor their content of HBV DNA, Southern blotting was performed with the same livers. As shown in Fig. 3B for two representative animals per group, the content of HBV replicative forms was not reduced in the livers of mice that received the cocktail of anticytokine antibodies (lanes 5 to 8) compared with the animals that received irrelevant antibody (lanes 1 to 4), in which the content of HBV replicative forms was substantially diminished at the same time point after 70% PH. When the same antibodies were administered separately, however, the PH-induced suppression of HBV replication was only partially blocked (not shown), suggesting that these cytokines may either function synergistically or activate independent antiviral pathways.

Based on the foregoing, it appears that 70% PH activates one or more antiviral pathways that transiently inhibit HBV replication in the hepatocyte, resulting in a decrease in nuclear and cytoplasmic HBcAg and replicative DNA intermediates. The loss of nuclear nucleocapsids after mitosis is consistent with their release into the cytoplasm as we have previously reported (10). The reduction of cytoplasmic nucleocapsids and viral DNA independently of liver cell regeneration is reminiscent of the inhibitory effect of inflammatory cytokines on these viral species in resting hepatocytes as previously reported (7, 9). Since TNF- α and IFN- α/β were induced in the regenerating livers, and since antibodies to these cytokines in combination with anti-IFN- γ antibody can completely block the PH-dependent suppression of HBV replication, it appears that inflammatory cytokines can exert their antiviral potential in dividing hepatocytes as well. Whether the cytokines inhibit core particle formation or accelerate particle degradation remains to be determined.

Most importantly, these results (Fig. 1D and 3B) demonstrate that both empty and mature core particles can survive in the cytoplasm of the regenerating hepatocyte if certain cytokines (i.e., TNF- α , IFN- α/β , and IFN- γ) are absent, indicating that HBV can efficiently replicate in dividing hepatocytes. The results also demonstrate that the expression of HBV RNA is not affected during liver regeneration in this transgenic mouse model, suggesting that HBV gene expression is relatively resistant to signals that upregulate or downregulate the expression of cellular genes during liver cell regeneration (3).

We thank Monte Hobbs for providing the cytokine probe set used in the RNase protection assays, Robert Schreiber for providing the hamster monoclonal antibodies to IFN- γ and TNF- α , Ion Gresser for providing the sheep antiserum to IFN- α/β , Susan Kramer for providing recombinant TGF- β , Margie Pagels for preparation and staining of tissue sections, and Rick Koch and Violeta Alvarado for excellent technical assistance.

This work was supported by grant R37CA40489 from the NIH.

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